CELL DIVISION AND DNA REPLICATION IN DIVISION MUTANTS OF *ESCHERICHIA COLI*

by

PETER MICHAEL LEIGHTON

Ph.D. University of Edinburgh 1971.

SUMMARY

Cell division and DNA replication in two mutants of *E.coli* have been examined. The *lon* mutant usually forms filaments after inhibition of DNA synthesis. A hypothesis is presented to explain this behaviour. The hypothesis is based on several assumptions but it can explain many of the findings of this thesis and serves as a good working model.

This hypothesis is as follows (i) that the *lon* mutation causes a defect in the cell envelope which, during normal growth, slows the rate of DNA replication (C ≃ 87 min) but does not otherwise affect the cell. (ii) that in both *lon*$^+$ and *lon*$^-$ cells, thymine starvation prevents the separation of the DNA from the cell membrane which normally occurs on termination. *Lon*$^+$ cells recover from this treatment if DNA synthesis is allowed to resume. In *lon*$^-$ cells, however, this inhibition of DNA synthesis prevents further septum formation which is hypothesized to occur at the site of DNA attachment to the membrane so that it can no longer act as a division site (the central division site of the cell). (iii) that during inhibition of DNA synthesis, an 'inhibitor' of division is formed which prevents division at other sites in the cell. This 'inhibitor' is formed in proportion to the length of inhibition of DNA synthesis with the maximum amount being produced after 60 min inhibition. Since the maximum inhibition of division lasts 120 min it is hypothesized that this is the time required for the 'inhibitor' to decay. Smaller amounts decay sooner, accounting for the shorter delay found with shorter periods of thymine starvation.

When division does occur after decay of the 'inhibitor', it does so at a site 2 - 3 'unit cells' (3.4 - 5μ) from one end. Inhibition of cell division by a treatment which does not affect DNA synthesis (low concentration of penicillin) does not cause filamentation of *lon*⁻ cells. This indicates that the *lon*-effect is linked to inhibition of DNA synthesis and not to inhibition of division.

The second mutation studied was the *min* mutation which causes small enucleate cells (minicells) to be divided off during normal growth. The nucleated mutant cells were found to be larger than wild-type cells and possessed fewer division sites than wild-type cells of the same length. This suggested that division sites in the mutant take longer to develop. No defect was found in DNA replication or cell division.

A comparison was made by gel electrophoresis of the envelope proteins of minicells and the large cells from which they separate. It was thought that the large cells might possess an extra protein peak which would represent the site of attachment of the DNA to the membrane. No difference in the profiles was found, and the significance of this has been discussed.

TABLE OF CONTENTS

CHAPTER 1 : INTRODUCTION

Possibly the simplest and most primitive form of cell division known is that of bacteria. Nevertheless, nuclear and cellular division must be coordinated in order that enucleate cells and cells with too much nuclear material do not arise. It is to these questions of cell division and its connection with DNA replication that this thesis is directed. Using two division mutants of Escherichia coli, the normal division process has been examined and cell division and DNA replication have been followed during and after various treatments which specifically affect DNA replication (thymine starvation, ultraviolet irradiation) and treatments which affect the cell in other ways (penicillin, pantoyl lactone). The relationship between cell division and the cell envelope* has been examined using chemical treatment and acrylamide gel electrophoresis, and the possibility that the division mutants used in this work are changes in the cell wall has been discussed. Finally, an attempt has been made to see what conclusions can be drawn about cell division in E.coli.

Development of the Cell Theory:- That bacteria multiply by cell division is accepted by anyone who looks through a microscope. What is not realized is that little is known of this important process, and what is known is of very recent discovery. Before looking at the beginning of our understanding of cell division, I will trace the development of the cell theory.

*For this thesis, the envelope is defined as the cell wall - cell membrane complex (133).

Only a dozen years ago, it was possible to ask "... is it legitimate to extend the term 'nucleus' to the Feulgen-positive particle in a bacterium? Is it legitimate to refer to a bacterial 'cell'? And moreover, in view of the absence of many components discriminated in bone fide cells, are we prepared to recognize the Feulgen-negative material in a bacterium as 'cytoplasm'?" (112) Now all three terms are used unthinkingly with reference to bacteria, and " ... the passage of time so shifts the basis of discussion that questions once of passionate interest come to require no reply and are dropped, unanswered, from the common field of interest" (112).

Only plant and animal cells were large enough to be seen in sufficient detail in the microscopes of the early biologists to study their internal composition. Not until the end of the nineteenth century were bacteria included in the by then accepted (though not firmly formulated) cell theory.

That cells (the definition of cell varying through the years) exist has been known for three hundred years. Hooke, from his observations in 1667 saw cells as simple containers. Further progress was slow, however, as may be seen from a report by Home in 1818 that muscle and nerve fibres are "formed by the joining together of red blood corpuscles in lines" (10). The first 'cell theory' was outlined by Schwann in 1839, based on Schleiden's observations of the previous year. In it he says "A common principle of development is the basis of all organic tissues,

however diverse they may be namely, cell formation; that is to say, nature never joins the molecules together directly into a fibre, tube etc. but always first fashions a cell or first transforms this cell, where necessary, into the different elements of structures as they occur in the adult state" (10, 121).

Spherical aberration of the microscope lenses caused much difficulty in the study of cells at this time. When Lister developed a microscope which corrected this problem, many of the 'cells' formerly observed were found to be haloes around small particles (10).

One idea for cell multiplication put forward by Turpin in 1826 was that cells begin as globules. This globule becomes hollow and is transformed into a vesicle. New globules arise from the walls of this vesicle and increase in size until it bursts releasing a new generation of globules to go through the same cycle (19). Cell division was obviously still unknown in 1846 when von Mohl wrote, "wherever cells are going to be formed, this viscous fluid (Protoplasma) precedes the first solid structures that indicate the future cells ... the development of structure in the substance is the process that initiates the formation of the new cell" (11). In 1853, T.H.Huxley tried to discredit protoplasm as the 'fundamental living substance' and the cell theory as a whole, although fifteen years later, at an address in Edinburgh, he described protoplasm as the "basis of physical life" (11).

Cell division was not without its supporters. In 1841, Remak observed division of embryonic red corpuscles and believed this to be the only method of cell proliferation. Von Kölliber stated in 1844 that even in the embryo, all cells are derived by continuous division from the segmentation spheres (19). Nägeli gave support to these ideas in 1846 and 1849 when he stated his law that in plants, new cells are formed always at the expense of pre-existing cells, and each cell gives rise to two or more daughter cells by division (19).

It was held by some workers in the 1860's that a cell need not have a membrane or wall, and that a membrane might even be a sign of degeneration (12). However, Remak repeated in 1862 his conclusions that "in animals, as in plants, cell division occurs by the ingrowth of solid septa from the envelope into the protoplasm" (12).

As far as animal and plant cells were concerned, the battle was won, and by the turn of the century, few people supported the doctrine of free formation of cells.

The question of spontaneous generation in bacteria was discussed and fought in parallel with these studies on the cell theory. The existence of bacteria as the agents of disease was suggested in 1546 by Francastorius (145), however it rested to Leeuwenhoek, 130 years later, to actually see and describe microorganisms. Redi (1626-1697)proved that macroorganisms (maggots) were not spontaneously generated by showing that if a jar containing meat was covered

with gauze, no maggots developed in the rotting meat (111, 145). However, microorganisms were thought to be an exception to this rule. Spallanzani found (1765-1776) no organisms grew if he heated the beef broth and then sealed the containers, and he pointed out technical errors in the experiments of Needham who did find bacterial growth after heating his broth. In turn, Needham claimed Spallanzani's experiments had changed the conditions such that spontaneous generation was not possible. Schulze (1836) passed air through acid solutions into boiled infusion and Schwann (1837) passed the air through red-hot tubes. Neither observed any microbial growth, but the claim was still made that the air had been affected. An experiment by Schroeder and von Dusch (1854), who passed the air through cotton filters into the heated broth and did not observe spontaneous generation still did not convince everyone (111, 145). Finally, in 1864, Pasteur, in his famous experiment with the gooseneck flasks showed that cultures could be maintained sterile if dust from the air was allowed to settle in the gooseneck. So, by the end of the nineteenth century, all life was recognized to arise from existing life and all cells arise by binary fission of preexisting cells.*

Development of understanding of the Nucleus:- A similar debate on the cell nucleus was carried on in parallel with that on cell division. The first mention of nuclei, as we understand the term, was in 1700 when Leeuwenhoek wrote to the Royal Society concerning his observations on the blood corpuscles of salmon and flounder (10). After this, they were observed in the blood cells of other animals, and in 1781, Fontana described nuclei in eel

*the budding of yeasts is, of course, an exception to this latter rule, but they still arise from preexisting cells.

epithelial cells, the first observation in other than blood cells. Not until the 1830's was it realized that most, if not all, plant and animal cells are nucleated (11). Even then, however, knowledge of this organelle was poor, and Schwann believed that nuclei were formed by a process resembling crystalization in a structureless fluid. A membrane then developed around the nucleus and the cytoplasm appeared within this membrane (10, 112). However, in the 1840's, Gunsberg and Breuer described nuclear division in pathological tissue (19) and its relationship to the cell cycle became established.

The question of whether bacteria possess a nucleus is very recent, and in 1952, Cameron, in his book "Pathology of the Cell" wrote, "Bacteria and blue-green algae have been reported as non-nuclear organisms, but they may have (my underlining) nuclear substance in solution or present as granules so finely divided as to escape attention" (19).

That bacteria may contain nuclei was first suggested by Cohn in 1875 and this was followed by many confirmations of his observation (19). In 1924, using their new stain specific for DNA, Feulgen and Rossenbeck reported that bacteria gave a negative Feulgen reaction. The following year, however, Voigt found a small amount of Feulgen positive material in thick smears of bacteria (19). In 1937, Stille and Piekarski, using the Feulgen stain clearly demonstrated nuclei in bacteria, but not everyone was convinced, on the grounds that the reaction is non-specific (16, 92). However, the results of Robinow in 1942 and 1944 (122, 123) using the HCl-Giemsa

stain of Piekarski and the accumulation of electron microscope evidence left no doubt.

The Mechanism of Cell Division:- Due to the lack of satisfactory techniques, the actual mechanism of cell division has proved difficult to study, and we still do not fully understand it. Initially the Gram-positive rod (Bacillus sp.) was used for studies, presumably because the cells are larger and the cell wall easier to stain. Although once thought to be different, the mechanism of cell division in Gram-positive and Gram-negative rods now appears to be very similar (see below).

In Bacillus sp., division is a three stage process. First there is growth of membrane dividing the cell in two. (Although the septum* is always drawn as a line across the cell, it should be remembered that it is really a disc formed by membrane growing into the centre of a circle from the circumference. For this reason, it must be a complex set of reactions.) When the cytoplasm has been divided in two by the new membrane, the cell wall grows in from the edges forming a strong wall between the two cells. The third stage is the splitting of the wall in two starting at the periphery. This is presumably an enzymatic process, allowing the cells to separate, and may occur some time after completion of the cross wall (P.Highton, pers. comm.).

At one time, E.coli was thought not to form a septum. Instead the membrane and wall invaginated together in a "constriction involving all layers at once as if a noose were being tightened around the equator of the cell" (152). It is now thought likely,

* a septum here refers to the invagination or ingrowth of the cytoplasmic membrane,possibly with inner-layers of the cell wall associated with it.

however, that like Gram-positive rods, E.coli (and presumably most Gram-negative rods) does form a septum (152). Clark (23) has provided further evidence for septum formation by showing that 5-7 min. before physical separation, the E.coli cell is divided into two compartments. After the septum has formed, it separates into two layers between which the cell wall invaginates. Finally the cross wall splits to release the two new cells (152).

This membrane is seen most easily if the cells are grown at 45° and fixation for the electron microscope is carried out at the same temperature (152). However, at room temperature (21° - 25°), a dark line can be seen across the cell 5 minutes before the appearance of the constriction at the same place. Also, using newer techniques of fixation for the electron microscope, membranous septa have been seen in cells grown at 30° (152).

Although the mechanism of DNA replication has been studied in detail, little work has been done on the parallel process of cell division. Little is known of the control mechanism for cell division, although it is likely to be linked to DNA replication. Smith and Pardee (147) reported accumulation of a heat-labile protein needed for cell division. This protein accumulates during the cell cycle and a heat shock synchronizes the following two cell divisions by delaying division in the older cells longer than in younger cells, with a maximum delay just before division of 25 min. The lability (or requirement) of this protein is lost about 4 min. before physical separation of the cells, and the authors suggest that the protein concerned is the septum itself.

Using time-lapse photomicrography, Hoffman and Frank (70) found that visible invagination occurred five minutes before cell separation. Also, Steed and Murray (152) reported that a membranous septum is found under certain conditions five minutes before visible constriction occurs. This gives a minimum figure of ten minutes from visible septation to cell separation. It is possible that the protein of Smith and Pardee is the membranous septum which does not become visible for 10-15 min and once it is complete, five minutes before invagination occurs, it is no longer thermolabile.

Bazill (13) described division in Bacillus as occurring when the rate of wall synthesis during elongation failed to keep pace with membrane synthesis. The membrane was then forced to invaginate and this was the first stage of cell division. This theory is unlikely since if it is to cause the wall to follow it across the cell, one would expect their rates of synthesis to be coupled.

On the other hand, the filamenting activity of penicillin might be used to argue that the cell wall initiates septation. Penicillin has the effect of blocking cell wall synthesis (151). At very low concentrations, it allows elongation but not septation (151). This would suggest that the bacterial cell wall possesses a very sensitive site which controls bacterial division.

Cell Growth:- Donachie and Begg (36) proposed a model for bacterial growth and division. There have been previous studies using fluorescent-labelled antibody (27), however, this method

examines cell growth only in terms of the outermost layer of the cell. It is not necessary that the method of cell wall growth be the same as that of the underlying cytoplasmic membrane. If, as is likely, it is the cell membrane which receives stimuli from the cell and co-ordinates cell growth with DNA replication, the growth of the membrane would be the more interesting and more relevant parameter to study. Jacob, Ryter and Cuzin (83) tried to do this using tellurite which attaches specifically to the membrane and can be observed under the electron microscope. One disadvantage of this method, however, is that tellurite is toxic and so may affect the cell division mechanism.

Jacob, Brenner and Cuzin (82) put forward a model for cell growth which assumed that the membrane grew only in the central area of the cell between the points of attachment of the two sister chromosomes to the membrane. However, no experimental evidence was put forward at that time to support this theory, although the later work with tellurite appeared to confirm it (83).

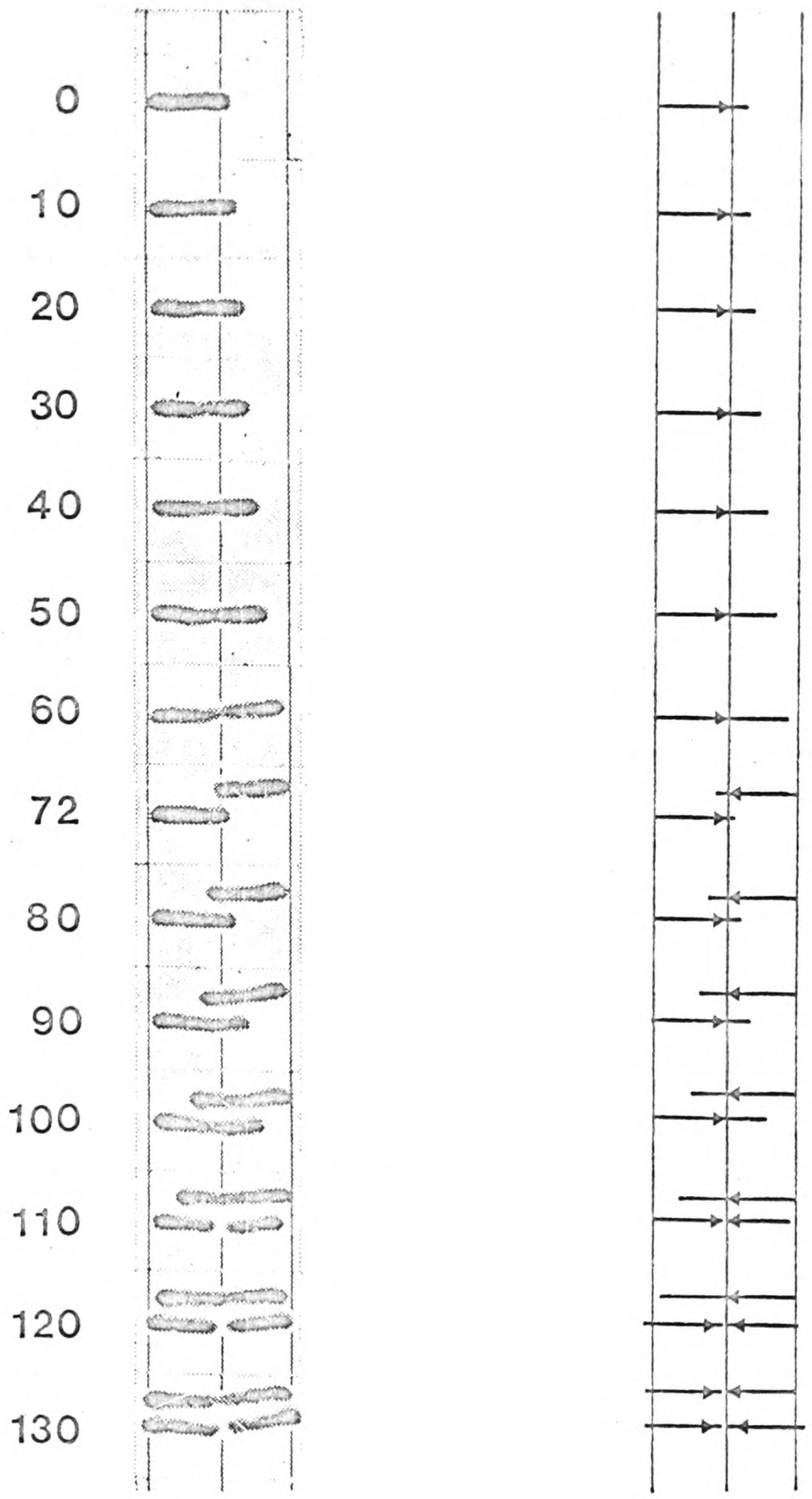
Donachie and Begg found that the pattern of cell elongation in E.coli varied with the growth medium. They interpreted their observations as indicating that in fast growing cells, new membrane is layed down in the central area of the cell. In minimal-grown cells, new membrane is synthesized from a growth point one cell length from one end and in a fixed direction away from that end. Their method was to measure the direction of growth of living cells with respect to external markers in the medium, and to an internal marker of the membrane. In minimal medium, they found that the cells grew unidirect-

ionally. When they were two cell lengths long, they divided and the 'new' ends now became the growing ends (36) (see Fig.1.). In rich medium, the cells were found to grow by elongation at both ends.

The dependence of the method of elongation on the growth medium is explained by means of growth sites and the 'unit cell'. A unit cell (which is assumed to be the smallest possible viable cell) has a growth site at one pole formed at the previous division. Growth is from this pole until the cell is two 'unit-cell'-lengths long with the growth site in the middle of the cell. The growth site duplicates and if at this time division occurs through the middle of it, then each cell will have a growth site at the new end, but growth will be in opposite directions in the two cells. In rich medium, the cells are longer and possess 2 growth sites, one 'unit cell' length from each pole causing growth in opposite directions with new material being laid down in the central area. This model, while it is a development of the model of Jacob et al is not the same as it. The results of cell growth in binucleate cells supports either model. However, in the model of Jacob et al, the membrane attachment sites of the sister strands of DNA are separated during growth. In Donachie's model, this need not be the case and the sister strands are separated by division.

There is one difficulty, however, which has not been explained by this model. This is the observation of Adler et al (3) that in cells growing on nutrient agar, the two pairs of nuclei can segregate with both sisters going to one new cell, or one of each pair

Fig. I



The growth of a single cell of *E. coli* 15T⁻ JG151 on minimal agar (phase contrast pictures of living cells). The positions of the ends of the cells were measured at intervals, relative to fixed markers in the agar, as described in the text. The numbers on the left are the times (in min) at which the adjacent photographs were taken. The three vertical reference lines show the positions of the left end of the cell, of the division site and the right end of the cell at the time of division. The cell grows by elongation of one end from 0–60 min. It divides between 60 and 72 min. The daughter cells then slip out of alignment and each continues to grow unidirectionally from the end which was formed at the division. At 110 min one of these daughter cells divides and at 130 min the other, slightly slower growing cell, also divides. (At the second division the proximity of the sister cell prevents the two halves of the newly divided cell from slipping past one another. Subsequent growth therefore pushes the outer ends of the cells over the reference lines.) On the right of the figure, lines are drawn to represent the length and position of each cell. The arrowheads represent the position and direction of growth of the proposed growth sites (see text). The vertical reference lines are each 1·7 μm apart.

from Donachie & Begg (36)

going to each new cell. The model of Donachie would be more in line with the first of these possibilities. In order to allow both possibilities, one must postulate that the DNA can separate from the membrane and random segregation occur. Conceivably, this is the reason for the 20 minute 'D time' between the end of DNA replication and cell division (see next section). This segregation must occur at the end of chromosome replication irrespective of whether division follows or not since segregation in a multinucleate filament would present difficulties. It must also be a very ordered process to ensure that the DNA is divided equally between the daughter cells.

Although the chromosomes appear to segregate randomly, this does not appear to be true for a chromosome and its episome. Cuzin and Jacob (29) found that parental copies of the chromosome and F were still associated after several generations. This would suggest that either the episome is somehow bound to the chromosome, or that when the chromosome separates from the membrane (assuming it does), a protein joining the chromosome and episome attachment sites separates from the membrane also, so keeping the two replicons together.

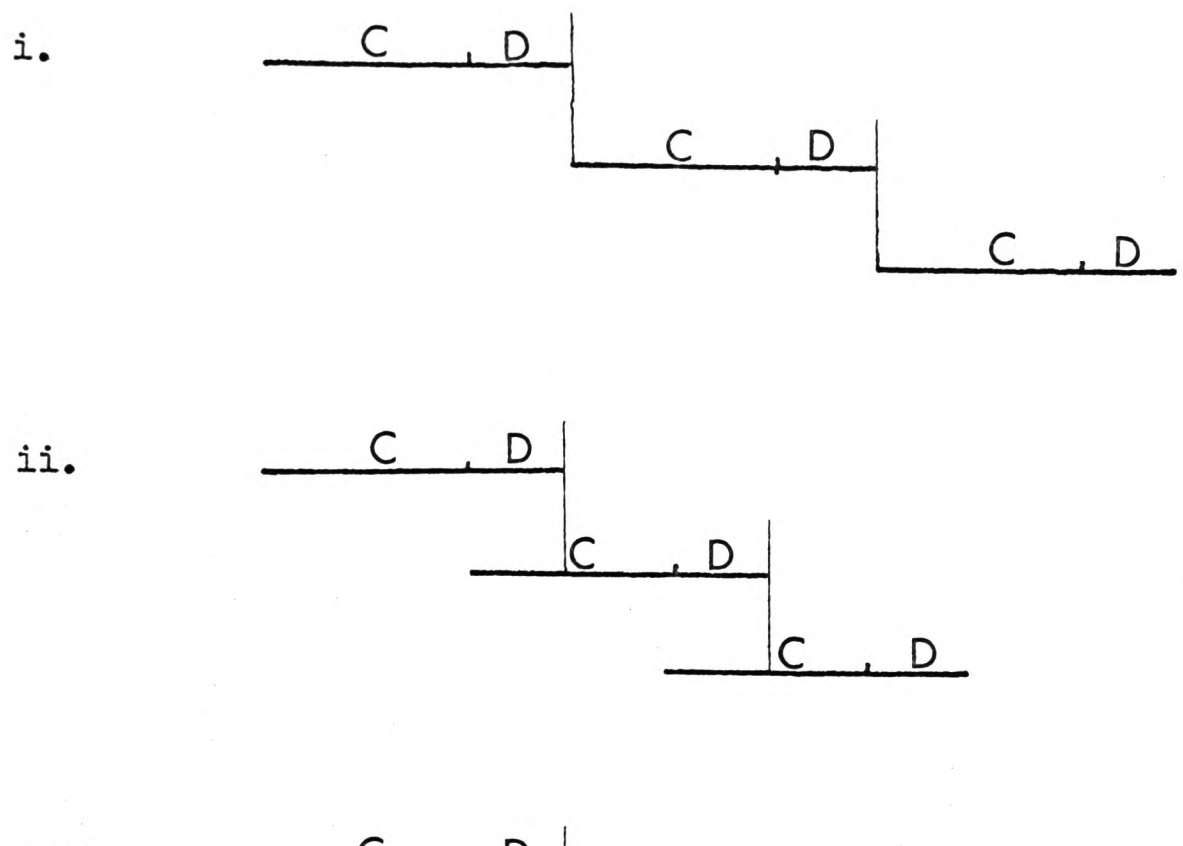
The Cycle of Cellular DNA Synthesis:- The DNA replication cycle in E.coli was characterized by Helmstetter and Cooper (28, 65) in a model which is now regarded as being largely correct. In this model, the cycle of chromosome replication was divided into two periods, C and D. C represents the time needed to replicate the chromosome, and since the polymerase is believed to travel at a constant

rate, for each strain of E.coli C is a constant. D represents the minimum time required to elapse between the end of replication and the consequent division. D, like C, is constant for growth rates between 1 and 3 doublings per hour. In their experiments with E.coli B/r, Helmstetter and Cooper found C to be 41 minutes and D to be 22 minutes (28, 65). Therefore, if the cell is growing with a generation time of 63 minutes, one cycle of chromosome replication equals one generation time. For growth rates faster than 63 minutes, rather than C or D being shortened, another round of replication is started before the cell cycle is completed. This means that for cells with a 40 minute generation time, 40 minutes after one initiation, another one starts and DNA synthesis is continuous. For growth rates faster than 40 minutes, a new round of replication starts before the old one is finished and dichotomous chromosomes result (see Fig II). In the work of this thesis, growth rates were maintained around 60 minutes to avoid dichotomous replication which could conceivably have made interpretation of the results of cell division more complicated. For generation times greater than 60 minutes (i.e. slow growing organisms), both C and D appear to be lengthened and D becomes one third of a generation time (28, 65, 66). For very slow growing organisms, there may also be a delay period at the beginning of the cycle before replication begins (28).

The generation time of a cell is determined by the time required to develop the capacity to initiate (I_t*) (66, 113). The

*Although Helmstetter et al (66) call the time required to develop the capacity to initiate I, I will call it I_t to differentiate it from the initiator protein of Pritchard et al (115) which is also called I (see below).

Fig II



Chromsome replication and cell division according to the scheme of Helmstetter and Cooper (28, 65). C is the chromosome replication time (40 min), D is the delay between termination and the consequent division (20 min). The vertical lines represent division. Generation times are i. 60 min, ii. 40 min, iii. 30 min. In i, DNA replication commences immediately after division and ceases 20 min before the next division. In ii, as soon as a chromsome terminates, the two daughter chromsomes begin. In iii, each of the partially replicated daughter chromsomes reinitiates before its replication is completed. On division, each cell receives a 75% replicated chromsome which immediately reinitiates at the two origins.

faster the cell is growing, the shorter is the time I_t and initiation is more frequent. This is a positive control mechanism and initiation occurs when a unit of initiator has accumulated per chromosomal origin.

The first formal model for this control mechanism was proposed by Donachie (34). He suggested that initiation of chromosome replication occurred when the cell mass reached a certain critical value (M_i). At faster growth rates, this critical mass is reached faster and the frequency of initiations increases.

Pritchard et al (115) developed the theory of initiation further but they hypothesized that there is negative control of initiation. They suggested that initiator (I) is produced constitutively. There is also an inhibitor (H) of initiation, the gene for which is part of or next to the origin and is transcribed only at replication. This gene produces a fixed number of mRNA molecules from which a fixed number of inhibitor protein molecules are translated at all growth rates. The inhibitor is diluted by cell growth until at two cell volumes, it is diluted enough that initiation can occur. When initiation occurs, another unit of inhibitor is produced which prevents further initiation.

The theory that initiation is controlled by the accumulation (or dilution) of a protein received further support from the findings that the ability to initiate chromosome replication is attained during inhibition of DNA synthesis. This ability is acquired at the cell age at which initiation would have occurred had DNA synthesis not been blocked (168, 169). But initiation is not completely independent of

DNA synthesis. For brief periods of DNA inhibition, so that the cell mass for initiation (M_i of Donachie (34)) is not reached during starvation, the next round of initiation is delayed by a time consistent with that needed to complete the round of synthesis in progress (168).

Lark and Renger (94) suggested that initiation of chromosome replication required the synthesis of at least two 'amino acid-containing moieties'. The synthesis of one of these is resistant to chloramphenicol. Ward and Glaser (167) also found initiation to be controlled by a chloramphenicol sensitive and chloramphenicol resistant step, but they disagreed with Lark and Renger as to the timing of the two steps. Lark and Renger found neither of the steps occurs at the time of initiation, but rather the sensitive and resistant steps occur 30 min and 15 min respectively before reinitiation. Since neither occurs at the actual time of reinitiation, a third unknown step not requiring protein or RNA synthesis was hypothesized. Ward and Glaser (167), however, found that the sensitive step occurs 21 min before actual reinitiation (a figure close to the result of Lark and Renger) and the resistant process occurs at the start of chromosome replication. A third stage is thus not required. This discrepancy may be explained by the fact that Lark and Renger were studying *E.coli* $15T^-$ and Ward and Glaser were using *E.coli* B/r.

Role of Membrane in DNA Synthesis and Cell Division:- In their model for DNA replication, Jacob, Brenner and Cuzin (82) suggested that in bacteria, the chromosome is attached to the cell membrane. Using serial sections of *B. subtilis*, Ryter and Jacob (131) showed that

the nucleus of the cell is always associated with a mesosome*. This view was later modified by them, and the nucleus is now believed to be attached to the cell membrane where it forms a sac around the mesosome (130). Gram negative organisms do not contain such well developed mesosomes, although they do contain intracytoplasmic membranes, to which the nucleus may be attached (114, 130, 132). Whether these membranous invaginations are mesosomes or not is open to discussion (130) and may be a matter of semantics. But this should not detract from the fact that they are cytoplasmic membrane and that they appear to be in contact with the nucleus (21, 55, 130).. They may therefore play a role in chromosome replication†.

Biochemical studies of DNA-membrane interaction confirmed the electron microscope findings. Using centrifugation, the replication (growing) points of E. coli (146) and B. subtilis (57) were shown to be attached to cell membrane. If this is the case, then it should be possible to isolate a complex containing membrane, DNA and RNA. Using the property of membrane to attach to crystals of detergent, Tremblay, Daniels and Schaechter (156) found such a complex from B. megaterium. The growing point of B. subtilis (81) and E.coli (56) were also isolated attached to membrane. Pulse labelling of E. coli with ^{3}H-thymidine yielded maximal labelling of membrane-bound DNA around the time of cell division. This suggested

* Mesosome is the name suggested by Fitz-James (52) for dense areas of intracytoplasmic membrane found in Bacillus sp.

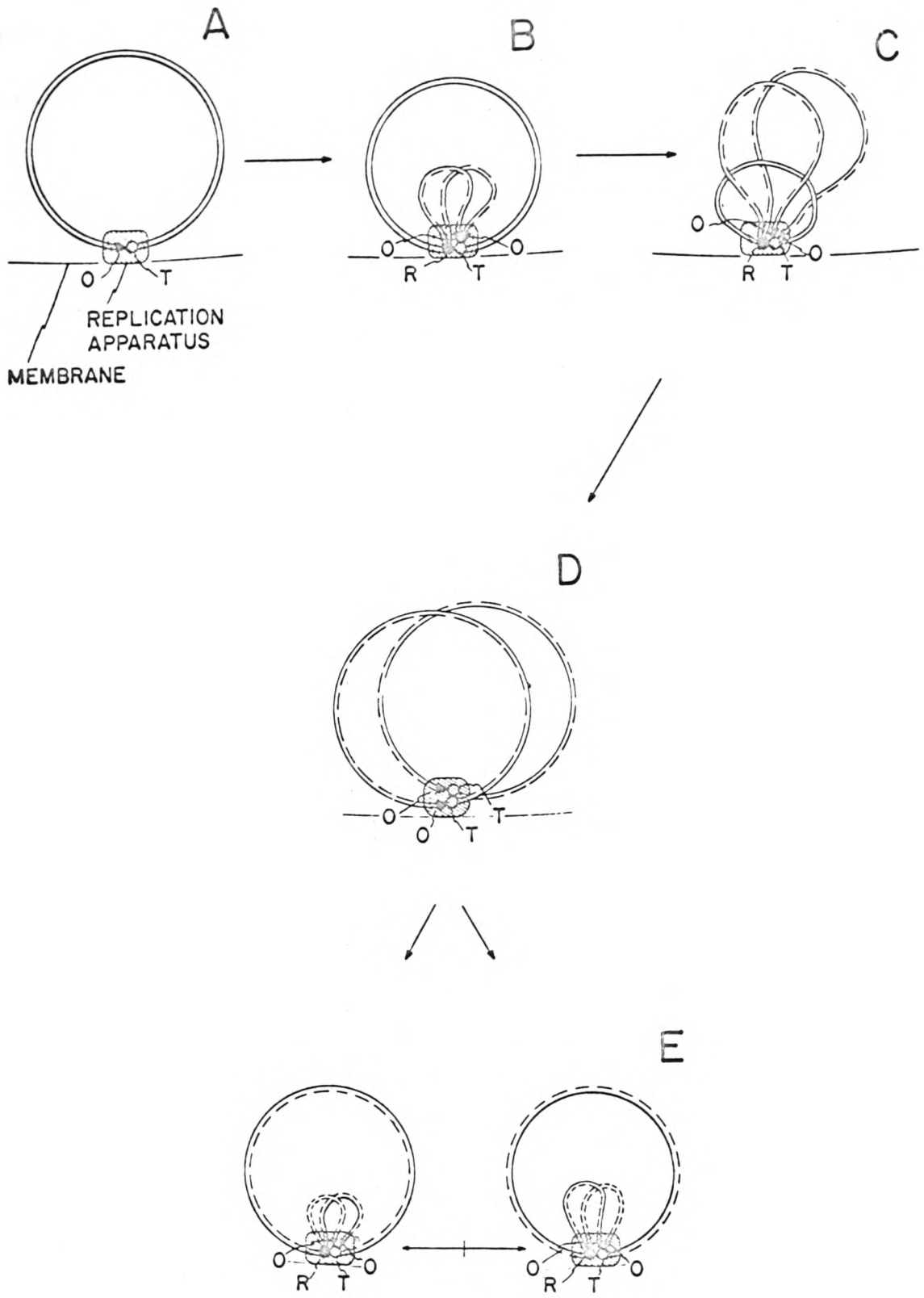
† For this reason, in the same way as the term nucleus has been broadened in its meaning to include bacterial DNA as well as the membrane-enclosed structure of higher cells, so, on the assumption that their function is the same, the simple membranous invaginations of Gram-negative organisms and the more structured intracytoplasmic membrane of Bacillus sp. will both be called mesosomes.

that apart from the replication point, the origin (or terminus) of the E.coli chromosome is attached to the membrane (50). This confirmed earlier results of Sueoka and Quinn (153) who proposed a new model for DNA replication to account for both the replication point and the origin and terminus of replication all being attached to the membrane (Fig III).

On the other hand, by electron microscopy, the chromosome of E.coli (114) and B. megaterium (46) appears to be attached to the membrane via two mesosomes. In both cells, one mesosome is at the pole of the cell and the other at the site of the cross wall. Similarly, B. cereus regularly contains 3-5 mesosomes per cell, with one mesosome at the division site in 96% of the cells (J. Garland, pers. comm.). B. subtilis has been reported as having only one (69) or several (131) mesosomes per cell. With Bacillus sp., therefore, it appears to be very much a species variation as to how many mesosomes a cell possesses, although it cannot be ruled out (especially in view of the contradictory results with B. subtilis) that the method of preparation for the electron microscope may determine what is seen. It has also been reported (44) that in Micrococcus radiodurans, if the cells are treated with 0.02M spermidine before fixation with osmium teroxide, the DNA shows multiple attachments to the membrane. Whether this is an artifact or not is not yet known.

Chromosome replication is likely to have some control over cell division to ensure that division does not occur before the genetic material has doubled. The two must, therefore, be intimately related, and a membrane site would provide a suitable point of contact. Mesosomes

Fig. III



Schematic representation of the replication process in the present model (Fig. 8C above). The process, A to D, completes one cycle of replication for one chromosome. Note in the diagram that the separation of the two daughter chromosomes proceeds simultaneously with replication, thereby making the replication continuous from generation to generation. At the termination of each replication cycle, the two daughter chromosomes are connected at the replication apparatus (D). The state A, therefore, could be attained only if the separation occurs without concomitant replication of the daughter chromosomes.

from Sueoka & Quinn (153)

in Bacillus sp. and E. coli are found at the division septa (46, 53, 114, 124, 131) and at division, the mesosome splits with one half going with each new nucleus. This makes it very likely that the completion of DNA replication causes some change in the membrane such that it is initiated to form a transverse septum. Donachie, Martin & Begg (submitted for publication) found that B. subtilis does not possess this control and division occurs in the absence of DNA replication.

Effect of Ultraviolet Irradiation on the Cell:- Ultraviolet (UV) irradiation of UV resistant cells causes temporary inhibition of DNA synthesis (86, 87, 90) due to the formation of pyrimidine dimers (primarily thymine dimers) (141, 142, 143). DNA replication can pass by the dimer, but each dimer causes a delay of about 10 sec (128). This figure is based on calculations by the authors that at 2537Å, 1 erg/mm^2 yields 6.5 dimers/10^7 nucleotides (one genome). At 45 erg/mm^2 (270 dimers/genome), the irradiated cells took 45 additional minutes to incorporate a given amount of ^{3}H-thymidine yielding a delay of 10 sec/dimer. On the other hand, Swenson and Setlow (154) found that UV irradiation yielded about one half this number of dimers. At 2650Å, 2 erg/mm^2 yields 1 dimer/200μ. Using Cairns (18) result of 1100μ for the length of the E. coli chromosome, and the assumption that the yield of biologically significant photoproducts is 25% higher at 2650 Å than at 2537Å (28), this would mean 5.5 dimers/genome but at about 1.6 times the dose.

UV-irradiation causes other damage to the cell apart from pyrimidine dimers. Cross-linking between DNA strands has been demonstrated, but it is not likely that it causes any biological damage

(43, 149). Cross-linking of DNA to protein has also been found, and although its role in causing biological damage has not been determined, it may well contribute to the lethality of UV irradiation (43, 149).

The cell contains two independent repair mechanisms to remove the pyrimidine dimers. The excision repair system is controlled by the uvr genes (71, 73, 74, 159). The pyrimidine dimer is excised, the gap enlarged slightly and the gap then filled by repair synthesis (177). This system is efficient at repairing damage due to high UV doses, but is inefficient for that due to low doses (118). For low intensities of UV, recombination repair controlled by the rec genes (22, 47, 77) is effective. There is no inhibition of DNA synthesis, and repair occurs during or after replication (118). The mechanism of action of these genes has not been determined, but a postulated mechanism for this repair is that the intact strand is used as a template for both daughter strands (72, 177). This has not been experimentally shown, however. It has also been suggested (58) that the rec-repair system can only repair gaps in the DNA and is, therefore, not active until after a gap has been produced in the sister strand by replication past the dimer.

After a high UV dose (when the uvr genes are responsible initially for DNA repair), DNA replication is inhibited. This is critical, since replication through the dimer would result in a gap in the complementary strand and a lethal double strand break would occur, either due to subsequent excision of the dimer (118)

or by replication or attempted replication of the discontinuous strand (46). The delay in DNA replication allows the cell to excise most of the pyrimidine dimers and repair the gap with the complementary strand as a template. When replication restarts, any remaining dimers can be removed by the recombination system.

More recently (162) another gene (ras) affecting UV sensitivity has been found, but apart from the theory that it is involved in the repair of pyrimidine dimers, its function is unknown. Cells mutant at this locus have normal recombination, can reactivate UV-irradiated phage and possess an increased rate of induced mutation.

Apart from these genetic mechanisms for the bacterial cell to recover from UV irradiation, various physical treatments can also aid their survival. Liquid holding, that is keeping the cells temporarily in a non-nutrient medium, aids their survival, presumably by slowing down the bacterium's metabolism and allowing more time for the repair of the DNA (121, 140).. Incubation at 42° increases survival in a converse manner by speeding up DNA repair more than other metabolic processes (9, 175).

Irradiation with light of longer or shorter wavelengths also increases survival of bacteria after UV irradiation (2200-3000Å, usually around 2500Å).

(i) photoprotection - cells are irradiated with light of wavelength between 3100 and 3700Å. Light of this wavelength has two effects. It aids recovery by inhibiting RNA and protein synthesis without affecting DNA synthesis, but at high doses it causes cell

damage itself (84, 139). No enzymes are involved in this mechanism of recovery from UV. Rather, the increased survival is due to the longer time available to the cell to repair the damage.

There are two kinds of photoreactivation, direct and indirect.

(ii) direct photoreactivation - the reduction in UV killing due to subsequent irradiation of the bacteria with light at wavelength 3100-4000Å. Light of this wavelength splits pyrimidine dimers due to activation of an enzyme which attaches to irradiated DNA (139).

(iii) indirect photoreactivation - resembles photoprotection in that it may act by causing a delay in growth and division. However, the mechanism of both of them remains uncertain (139).

(iv) Yet another way to aid recovery of the bacterial cell from UV damage is to irradiate with light of a shorter wavelength. This is because light of different wavelengths possesses different energies and so affects the thymine molecules differently. Whereas light of 2800Å forms dimers, at 2390Å, the dimers are broken (33,142).

UV irradiation inhibits DNA synthesis. It has been reported that when chromosome replication restarts afterwards, it does so not from the old replication point but from the chromosome origin (67). The authors also stated (14, 15) that there was no increase in the rate of DNA synthesis after UV. This they explained by saying that apart from cessation (or severe reduction) in replication at

the old growth point, reinitiation occurred on only one of the two new strands.

However, the results of this thesis indicate that there is, in fact, an increase in the rate of DNA synthesis after UV-irradiation. The DNA doubling time was almost halved. This would suggest that there is reinitiation on both daughter strands.

Effect of Thymine Starvation on the Cell:- Although at first they would appear to be very different in their mechanisms of action, thymine starvation affects the cell in a very similar way to UV irradiation. Thymine starvation also causes random single strand breaks in DNA (54, 103, 110, 163) at the rate of 0.029 breaks/74 x 10^6 daltons/min at 37°C or 0.98 breaks/genome/min at 37°C (54). A metabolic step is needed for the breakage since it is not observed if the organism is incubated, during thymine starvation, in the absence of glucose, phosphate or hypoxanthine (the latter used to satisfy the adenine requirement of the E.coli strain used) (54). When the organism was starved of required amino acids during thymine starvation, there was a two-fold reduction in strand breakage, but it was not eliminated. Since the strain used was stringent with respect to amino acid control of RNA synthesis, it is unlikely that either protein or RNA synthesis is necessary for strand breakage (54). One possible cause of the single strand breaks is that nicks occur in the DNA during normal metabolism. These nicks are normally repaired by DNA ligase, but this enzyme is inhibited by the dATP accumulating during the thymine starvation, so that as metabolism continues, more and more nicks accumulate in the DNA (54, 110). For

very long periods of thymine starvation, some of the cells would accumulate more nicks than they could repair on readdition of thymine and the cells would die.

The removal of thymine from the medium often does not result in immediate cessation of DNA synthesis. Many strains contain a thymine pool, the size of which varies with the strain. E.coli $15T^-$ has a very small pool (20) whereas E.coli K12 appears to have a larger one (20). This has been confirmed by the work of this thesis. Thymine starvation of a K12 strain resulted in a lag of 20 min between removal of thymine from the medium and cessation of incorporation of thymine into DNA.

Thymine starvation disturbs the cycle of DNA synthesis. On the readdition of thymine to the medium, DNA replication starts again, but the rate of synthesis is greater and reaches a maximum rate of slightly over twice the prestarvation rate after 30-40 min of thymine starvation (116). However, treatment with nalidixic acid (a specific inhibitor of DNA synthesis) resulted in a greater than three-fold increase in the rate of DNA synthesis (115), suggesting reinitiation on both strands and continuation of synthesis at the old replication point. This was more in line with what was expected and what the authors had suggested in an earlier note (93). The lower rate of synthesis after thymine starvation could be explained by reinitiation occurring at all three sites, the old replication point and the origins of both daughter strands, in only 50% of the cells. In the remaining 50%, only the old replication

point continues (115). Another possibility is that some cells (33%) fail to replicate their DNA at all after thymine starvation (115).

Thymineless Death:- A brief word must be said about thymineless death since it must be differentiated from the death caused by thymine starvation of the lon - mutant to be described in Chapter 3. E. coli $15T^-$ is a thymine-requiring derivative of E.coli 15. When it was starved of thymine, there was found to be a decrease in the number of colony forming units (26) which was called thymineless death. UV irradiation had a similar effect (104). It was found to be due in part to the production by the bacteria of a lethal factor to which the bacteria were themselves sensitive (101, 129). This factor was presumed to be a colicin and so was called col_{15} (104). Production of this 'colicin' is triggered by the readdition of thymine to the medium (101) or by UV irradiation (129). However, electron microscope studies of cells induced to lyse by azaserine (135), mitomycin (48) and UV (102) showed the presence of phage and phage parts in the lysates, suggesting that the phage accounted for at least part of the 'thymineless death'. The possibility of a colicin still being present in addition to the phage was not ruled out, however, since cells were still found to die in the absence of phage induction. This death was shown to be a plating death (37). E.coli $15T^-$ JG.151 "col^-" does not produce a colicin and phage lysis and the plating effects were claimed to account for all the death observed after thymine starvation.

CHAPTER 2: MATERIALS AND METHODS

E. coli Strains: The strains used and their sources are given in Table I. The thymine-requiring strains TG894 and P678-T were derived from their parent strains by treatment with trimethaprim (courtesy of Dr L.G.Petty, Bourroughs Wellcome) (150). TG894LL was derived from TG894 by transduction with P1/kc W3110 and selection for *lac*$^+$ colonies, so that these two strains are isogenic with respect to the gene for thymine auxotrophy. All the thymine-requiring strains used showed a thymine requirement of 2 µg/ml. Most of the experiments with the *lon* strains (wild-type and mutant) were done in 10 µg/ml thymine. Subsequent information revealed that thymine requiring strains may have a reduced rate of DNA synthesis/ replication fork in the presence of less than 30 µg/ml thymine (117), and subsequent experiments were performed at this concentration. Similarly, the amount of thymine added to minicell cultures requiring it was increased from 10 to 30 or 50 µg/ml.

Media: Except where otherwise stated, experiments were performed in glucose minimal medium of the following composition: 300 ml distilled water, 100 ml M9 salts (4X)*, $MgSO_4$ (10^{-3} M), glucose (0.2%). To this medium was added 8 mg of each of the required amino acids (see Table I) and 8 mg vitamin B_1. Where necessary, thymine was added to the culture flask as described above.

* The composition of the M9 salts used is a variation of that given by Roberts *et al* (120). It had the composition Na_2HPO_4 (28g), KH_2PO_4 (12g), NaCl (2g), NH_4Cl (4g), distilled water (1 litre).

TABLE I

Strain	min	lon	thy	arg	his	thr	leu	pro	vitB1	Source
AB1157	+	+	+	-	-	-	-	-	-	H.I.Adler
AB1899NM	+	-	+	-	-	-	-	-	-	AB1157 (A.Hardigree)
AB2497	+	+	-	-	-	-	-	-	-	AB1157 (P.Howard-Flanders)
TG894	+	-	-	-	-	-	-	-	-	AB1899NM
TG894LL	+	+	-	-	-	-	-	-	-	TG894
P678	+	+	+	+	+	-	-	+	-	A. Hardigree
P678-54	-	+	+	+	+	-	-	+	-	P678 (A. Hardigree)
P678-T	+	+	-	+	+	-	-	+	-	P678
X806	-	+	-	+	+	-	-	+	-	P678-54 (A. Hardigree)

AB1157 has the genotype K12 $\underline{lon}^{+}$ $\underline{thr}^{-}$ $\underline{leu}^{-}$ $\underline{pro}^{-}$ $\underline{his}^{-}$ $\underline{met}^{+}$ $\underline{arg}^{-}$ $\underline{lac}^{-}$ $\underline{gal}^{-}$ $\underline{ara}^{-}$ $\underline{xyl}^{-}$ $\underline{mtl}^{-}$ $\underline{B1}^{-}$ $\underline{ton}^{s}$ $\underline{tsx}^{r}$ λ^{s} $\underline{str}^{r}$.

P678 has the genotype K12 $\underline{F}^{-}$ $\underline{min}^{+}$ $\underline{thr}^{-}$ $\underline{leu}^{-}$ $\underline{B1}^{-}$ $\underline{lac}^{-}$ $\underline{mal}^{-}$ $\underline{man}^{-}$ $\underline{gal}^{-}$ $\underline{xyl}^{-}$ $\underline{ara}^{-}$ $\underline{str}^{r}$ $\underline{Az}^{r}$ $\underline{ton}^{r}$ non-lysogenic.

For experiments requiring an enriched medium, minimal casamino acid medium (MCA) was used. This had the composition 300 ml distilled water, 100 ml M9 salts (4X), $MgSO_4$ (10^{-3}M), glucose (0.2%), vitamin B_1 (8 mg), casein hydrolysate (Oxoid) (0.2%), tryptophane (8 mg), and uracil (4 mg). Thymine, 10 or 30 µg/ml, was added to the flask where necessary.

The L-broth used in transduction consisted of tryptone (Difco) 10 g), yeast extract (5 g), NaCl (10 g) and distilled water (1 litre). The L-agar used in these experiments has the same composition except that 15 g agar was added (12 g agar for L-soft agar) as well. The L-agar also contained thymine (10 µg/ml) and $CaCl_2$ (2.5×10^{-3} M).

Minimal agar was used for all plating unless otherwise stated and consisted of 100 ml distilled water, 300 ml water agar (Davis)(2%), 8 ml VB medium (50X) (16l) and glucose (0.2%). To this were added 8 mg of each of the required amino acids (see Table I) and 8 mg vitamin B_1. Where necessary, thymine was added to the agar. When selecting for $\underline{lac}^+$ colonies, lactose was substituted for glucose and the thymine concentration reduced to 2 µg/ml.

Microscopic observation of growth and division was performed on glass slides covered with minimal soft agar. To minimize the shock of being transferred from liquid to solid medium, the agar concentration was reduced to 0.8%. The medium was prepared at 4X concentration and diluted as needed with three volumes of molten agar (1.1%).

The filament-inducing medium (F-medium) (172) had the composition: nutrient broth (Oxoid No.2) (2.4%), NaCl (0.75%), lysine (1%), and casein hydrolysate (Oxoid)(5%).

DNA Inhibition: For experiments involving thymine starvation, the culture was filtered on a Millipore filter (47 mm diameter, 0.45 μ pores). The filter was not allowed to dry and was washed with about 100 ml of prewarmed medium. The filter was removed from the filtration apparatus and placed in the flask containing thymineless medium. After vigorous shaking to dislodge as many bacteria as possible from the membrane, it was removed from the flask which was placed in the shaking water bath.

UV irradiation was carried out by means of a Hanovia bactericidal ultraviolet unit (Hanovia Ltd., Slough).

Cell Mass and Cell Number: To measure total cell number, samples were taken into 10 ml azide saline (0.9% NaCl, 0.08% NaN_3) and a 0.05 ml volume counted using a Coulter Electronic Counter (Coulter Electronics, Dunstable) with a 30 μ orifice.

In some experiments, penicillin was used to inhibit cell division. 'Solupen' (Dista Products Ltd., Liverpool), a commercial form of sodium penicillin G, was used at a concentration of 15μg/ml (25 u/ml). The penicillinase was derived from Staphylococcus aureus P.C.1. (courtesy of R.Ambler).

Increase in cell mass was measured by following the optical density at 540 nm using a Hilger & Watts spectrophotometer (Hilger & Watts Ltd., London).

Microscopic Study of Division: A glass microscope slide was covered with minimal soft agar. A loopful of the culture medium was placed on the agar and covered with a glass cover slip. The excess agar at either end was cut away, and the two ends closed with vaseline to diminish evaporation. The slide was then placed on a Wild microscope at 37° and observed using a 50X and 100X oil immersion lens. In order to minimize cooling of the bacteria, the slides were prewarmed at 37° and all operations were performed at 37°.

Sensitivity to Acriflavine: The organism to be tested was grown overnight in nutrient broth. Fresh, pre-warmed nutrient broth was inoculated from this culture to give an optical density around 0.05. After 90 min growth at 37° in a shaking water-bath, half the culture was removed and added to acriflavine to give an acriflavine concentration of 5 µg/ml. Both cultures were allowed to continue growing in the shaking water-bath for 2 hrs. Dilutions were then made in nutrient broth and 0.1 ml of the 10^{-4}, 10^{-5} and 10^{-6} dilutions plated on nutrient agar containing added thymine (50 µg/ml) (3 plates/dilution). After overnight incubation, the number of colonies was counted.

Sensitivity to sodium deoxycholate: The organism to be tested was grown overnight in L-broth. 0.02 ml of this culture was added to 2 ml of L-broth containing deoxycholate at the concentration 1%, 0.1%

and 0%. The optical density was measured at this time zero and after 4 hrs of growth.

Gel electrophoresis:

i. Preparation of gel: The gel contained 10% acrylamide with 0.5% cross-linking agent. A stock solution containing 20% acrylamide and 1% ethylene diacrylate in distilled water was prepared and kept in the cold. 5 ml of this solution was mixed in an ice bath with 4.75 ml of sodium phosphate buffer (pH 7.0, 0.2 M) containing 1% sodium dodecyl sulphate (SDS). The buffer-SDS had to be stored at room temperature to prevent the SDS coming out of solution. The mixture was degassed by suction for 15-30 sec. 25 µl of N,N,N,N-tetramethyl ethylene diamine (TEMED) and 0.25 ml of freshly prepared 10% ammonium persulphate were added in that order to the flask. After thorough mixing, the running tubes were filled using a syringe with a piece of thin tubing on the end to give gels about 18 cm in length (4 mm in diameter).

ii. Preparation of solubilized envelope from lon strains: 50 ml cultures of AB1157 and AB1899NM were grown overnight in minimal medium containing ^{14}C-leucine (20 µC/ml) or ^{3}H-leucine (100 µC/ml). The cultures were mixed and spun at 3000g for 10 min at 4°. The sediment was resuspended in 200 ml cold 0.1 M phosphate buffer, pH7.0, containing leucine (20 µg/ml) and respun. The sediment was resuspended in 8 ml 0.01 ml phosphate buffer, pH7.0 and lysed in a frozen state in an 'Eaton press' (45). The lysate was centrifuged at 3000g for 10 min at 4° to remove whole cells and large pieces of cell debris and the supernatent then spun at 100,000g for 30 min at 4°. At this

speed, the bacterial envelope sedimented to the bottom of the tube. The sediment was resuspended in 10 ml of buffer and respun. The envelope was suspended in 1 ml of solubilizing solution (mercaptoethanol (1%), SDS (1%), glycerol (10%), phosphate buffer (0.01 M) and bromphenol blue (0.002%)) for 20 min at 70°. Equal volumes of envelope solution and 60% sucrose were mixed and 0.1 ml applied to the top of the gel with a micropipette.

iii. Preparation of solubilized envelope from min strains: For the comparison of the cell envelopes of differently labelled 'mother-cells' of P678-54, the same method was used as that described above for lon strains.

For comparison of 'mother cells' with minicells, the centrifugation speeds had to be changed. Cultures were grown in the presence of ^{3}H-methionine or ^{35}S (sulphate) in 800 ml of minimal medium with the sulphate content reduced from 10^{-3} M to 2.5×10^{-4} M. The cultures were centrifuged at 3000g for 10 min at 4° which sedimented the cells. The supernatent was centrifuged at 10,000g for 10 min to sediment the minicells. The cells and minicells were each washed once and the minicells from one culture mixed with 1/13th of the volume of cells from the other cultures. After lysis, the mixture was centrifuged at 10,000g to remove all unbroken cells and minicells. Treatment was then as for lon cells.

iv. Running and Scintillation Counting of Gels:

The gels were run according to the method of Samson and Holland (134). The gels were run for 3 hr at 3 ma/gel and the current then increased

to 6m/gel for 9-10 hrs. The gel was removed from the tube after electrophoresis by introducing water around both ends of the gel using a syringe and needle. Extreme care had to be taken not to damage the gel since it was very brittle. The gel, freed from the running tube was placed on a thin aluminium tray in dry ice. The gels froze solid and could then be sliced. The gels had to be maintained in a solid state by keeping them covered with dry ice during slicing. The gels were cut into 1mm thick slices on a Mickle gel slicer and the slices dissolved in 0.5ml of 1.6M aqueous NH_3 at 37°. When the gel had completely dissolved, 10ml of scintillation fluid containing 2% Cab-O-Sil (Packard Instrument Ltd., Wembley, Middx.) was added. The scintillation fluid used was that of Pritchard and Lark (19) and had the following composition: naphthalene (80g), PPO (5g), absolute alcohol (216ml), 1,4-dioxan (360ml) and toluene (360ml). The samples were counted in a Packard Tri Carb Liquid Scintillation Counter.

Cab-O-Sil is finely divided silicon dioxide (171). It dissolves to give an optically clear thixotropic system. It can be poured, but, when stationary it forms a gel which prevents the settling of undissolved particles. If Cab-O-Sil is not used, on addition of the scintillation fluid, the acrylamide gel comes out of solution as a large mass. If the Cab-O-Sil is used, however, the system is perfectly clear.

Scintillation Counting of Filters: DNA synthesis was followed by the uptake of ^{14}C-thymine (the Radiochemical Centre, Amersham). ^{14}C-thymine (10 µg/ml, 0.1µc/ml (lon) or 0.2µC/ml (min)) was added

to the medium, and 0.5ml culture samples collected into 0.5ml of 15 or 20% trichloracetic acid (TCA) in ice. These were then filtered on Oxoid membrane filters (2cm diameter 0.45 μ pores). The filters were washed with 5% TCA and dried under an infra-red heat lamp. The dry filters were placed in vials containing 10ml 0.4% Scintillator BBOT (Ciba) in toluene, and counted in a Beckman Liquid Scintillation Counter.

CHAPTER 3: THE LON-MUTATION

SECTION I Introduction

The Lon-Mutation:- The lon-mutation was first isolated by Howard-Flanders (75) as a mutation in E.coli K12 causing sensitivity to ultraviolet irradiation. This mutation causes the cells to filament after UV-irradiation. Lon$^-$ cells showed increased sensitivity to ionizing radiation, and the colonies on minimal agar are mucoid (5, 75). The filaments produced by UV-irradiation can reach 100X normal cell lengths before lysis (8). The increased UV sensitivity is not due to a defect in the DNA repair mechanism since lon$^-$ cells are able to support the growth of UV-irradiated phage to the same extent as lon$^+$ cells (75). After irradiation, DNA and RNA synthesis and mass increase are normal (8, 32, 164). Nevertheless, the lon-gene appears to exert its effect on the cell through the DNA. Filamentation can be caused by nalidixic acid, hydoxyurea or 5-bromodeoxyuridine (BUdR)(88, 165) as well as irradiation. Thymine starvation can also induce filamentation in lon$^-$ strains (40, results of this thesis). These treatments all share the property of inhibiting DNA synthesis, suggesting that it is the inhibition of DNA synthesis which results in expression of the lon-phenotype.

The lon-phenotype can be prevented or reversed by various treatments e.g. by liquid holding (121), by growth at 42° (8, 9, 175) or by treatment with pantoyl lactone (5, 63, 160, results of this thesis). A common result of these treatments is a decrease in the

growth rate of the cells. Steed and Murray (152) have reported, however, that both growth at 41° and pantoyl lactone stimulate septum formation, but the mechanism of this stimulation is not known. It has also been reported (1, 51) that cell division can be induced by a cell extract. This extract does not appear to inhibit growth (H.I.Adler, pers. comm.) and its action is so far not understood. Streptomycin can reverse the lon-phenotype (144). At high concentrations (100 µg/ml) of streptomycin, the reversal is likely to be by inhibition of protein synthesis, however, at concentrations (1 - 1.5 µg/ml) too low to cause significant inhibition of protein synthesis, the lon-phenotype is still reversed for low doses of UV (less than 300 erg/mm^2).. The action of streptomycin is to cause misreading, mainly of pyrimidines, during translation on the ribosomes (30, 60). Low levels of misreading allow suppression of cell mutations, especially at sub-lethal levels of the drug in streptomycin sensitive cells. That this is likely to be the mode of action for streptomycin on lon cells receives support from the observations that lon-mutations are suppressible (97, 99) and streptomycin is able to reverse suppressible mutations (30).

The Fil-Mutation:- It has been known for a long time that E.coli B filaments after UV irradiation (59). This was one of the differences between B and B/r noted by Witkin (172). The name fil for the gene controlling filamentation in E.coli B was suggested by Rörsch et al (127). They suggested that B contains the wild type fil$^+$ whilst B/r is fil$^-$. As was later also found in E.coli K12 lon$^-$ (7), the filaments of B possess regularly spaced nuclei and nuclear division is normal (49). Extraction and analysis indicated

that the DNA (and RNA) content of filaments is the same per unit cell mass as for normally dividing cells (31, 32) and increase in mass after UV is also the same for both B *fil*$^+$ and B/r *fil*$^-$ (127). The *fil*-locus of strain B was thought to be different to the *lon*-locus which causes UV sensitivity and filamentation in K12 (62, 76, 85). In K12, filamentation is a mutant phenotype (*lon*$^-$) whereas in B it is wild-type (*fil*$^+$). Colonies of *lon*$^-$ cells were mucoid but colonies of *fil*$^+$cells were not. They were reported to map close but separately, and *lon* was thought to cause greater sensitivity than *fil*. However, *lon* and *fil* are in fact the same, as was shown by transduction of the *fil* gene into a K12 strain. The transductant was mucoid and filamented after UV irradiation (41).It was then found that B/r, rather than being *fil*$^-$, is *lon*$^-$ but possesses a suppressor of *lon* (*rad* (40) or *sul* (39)) which accounts for its UV resistance (*lon*$^-$ *rad*$^-$) whilst strain B is wild-type for this suppressor (*lon*$^-$ *rad*$^+$) (39, 40).

The Cap R Gene:- Yet a third name for the *lon*-gene (or one part of the *lon*-gene) has existed. Markovitz (96) identified a gene (R_1) linked to *pro*$_2$ (*proC*) which was a regulator gene for the synthesis of capsular polysaccharide in *E.coli* K12. The non-mucoid state (R_1^+) was dominant to the mucoid state (R_1^-) suggesting that R_1^+ produces a regulatory substance which is absent or defective in R_1^-. AB1899 was found to be R_1^- (96). A second regulator gene for capsular polysaccharide synthesis (R_2) not linked to *lac*, *pro* or *leu* was found (96). This gene was subsequently found to map near *trp* (98) and is now called *cap* S and does not affect radiation sensitivity (95). It was found that if either of these two genes was in the mucoid

state (R_1^- or R_2 mucoid) the organism was mucoid (96). The R_1 locus also determined UV-sensitivity (6, 98) and R_1^- strains formed filaments after ionizing radiation. These filaments could be induced by pantoyl lactone to form septa and divide (6). On account of these findings, and the possibility that mucoidity was needed for the lon-phenotype, a non-mucoid strain of AB1899 lon$^-$ (AB1899NM lon$^-$) was isolated (6) and it was found to retain the UV-sensitive, filamenting properties of the parent strain. Therefore, capsule formation is an effect of the lon-mutation which is not necessary for filamentation. This mutation to non-mucoidity is due to a suppressor or a mutation in a structural gene for polysaccharide (97). That R_1 (now called cap R) is the same as lon is very likely because of their phenotypic similarity (UV-induced filamentation (5, 6, 75), both able to reactivate UV-treated phage T1 or T7 to the same extent as wild-type cells (75, 97)) and closeness of mapping (5, 96).

Mode of Action of Lon:- The mode of action of lon is not known, although various ideas have been put forward. Initially all theories were based on the idea of UV-damage to the cell. The amount of irradiation required to inhibit septation is very low, and the target size is of the order of the whole chromosome (165). The damage was thought to be, therefore, the creation of DNA-protein cross-links (164). Another untested possibility is the accumulation of DNA precursors (165). It is quite conceivable that inhibition of DNA synthesis and the consequent accumulation of DNA precursors could act as an initiator for the lon-gene which would then produce

a repressor of cell division. If this were the case, the lon- gene could be a control gene to prevent cell division in the absence of DNA replication. In the mutant cell, this switch would perhaps be more sensitive or less easily reversed. Inhibition (or slowing) of protein synthesis after DNA synthesis has restarted would possibly cause the cell to use up its pool of DNA precursors rather than synthesizing them as it does during normal growth.

Prophage induction in K12 (λ) and filamentation share common inducers (174, 176). Both effects are initiated by UV or other treatments affecting the DNA; both occur occasionally in untreated cultures, especially 'old' cultures; they are photoreversible, that is they require the presence of pyrimidine dimers, although at the doses used, there may be only 10-20 dimers per bacterium; both are reduced by treatments reducing the rate of protein synthesis (174, 176). For this reason, Witkin suggested (174) that the lon- induced UV sensitivity of B was due to detachment of a phage from the bacterial chromosome and repair was reattachment of the phage. B/r would possess a more efficient mechanism for the reintegration of the phage. Treatment with acriflavine, which inhibits phage reintegration, causes the UV-survival of B/r to resemble that of B (174). The UV-survival curve of B possesses a break, indicating a resistant fraction of the population (see also UV-survival curves for lon$^-$ strains later in this chapter) which would represent organisms with increased efficiency of reattachment. This theory requires all B and K12 organisms to carry a prophage whose attachment is controlled by the lon locus. There has been no evidence to suggest

the widespread occurrence of such a phage. Witkin presented another theory (176) suggesting that B contains a repressor of lon. Lon produces a protein which inhibits cell division. UV causes inactivation of the repressor by a complicated process in the manner of the inactivation by UV of the λ-repressor. When the DNA is fully repaired, the (lon) locus is repressed again. Filamentation occurs if too much inhibitor of cell division has been produced to be diluted by growth of the filament before the filament becomes too long for the process to be reversible.

This theory received some support from results with K12 (λ) strain with a mutation (T-44) causing phage induction at 40° (91). When cured of its phage, this organism filamented at 40° and these filaments resembled those of lon in that they were prevented by the addition of pantoyl lactone. DNA synthesis and nuclear division in these filaments was normal. This does not mean that filamentation and prophage induction are the same process. Indeed, the dose of UV needed to induce C-600 (λ) (the parent strain of T-44) or AB1899 (λ) is about 200 erg/mm^2 which is about 100 times higher than the dose necessary to inhibit division of AB1899 (91). However, these results do suggest that phage induction and filamentation share a common factor.

Genetics of the Lon Locus:- When the lon-gene of E.coli K12 was first discovered, it was described as being between lac and gal on the E.coli chromosome map of Taylor (155) near the tsx locus (5, 75). This was in a similar location to that of the fil locus of

E.coli B (160). This received confirmation from Markovitz who mapped the cap R (R_1, lon) locus as being closely linked to proC (96). Donch and Greenberg (42) found that in fact the lon locus consists of two phenotypic classes, A and B. The order of the genes is:

thr leu proA proB lacZ proC tsx lon Class A lon Class B purE.

Organisms of class A are less sensitive than those of class B. AB1899 belongs to class A (42). Markovitz had previously described (97, 98) three phenotypic types of cap R mutants; capR6, cap R9 and cap R66 in the order, proC cap R6 cap R66 cap R9. They were selected from non-mucoid F'13 bacteria. Cap R9 and cap R66 show dominance for mucoidity when on the episome and recessiveness when on the chromosome, however, UV resistance is dominant regardless of position on the episome (158). Cap R66 and cap R9 show similar UV sensitivity and appear to be class A (42, 97). Cap R6 is more resistant than either class A or B. However, these mutants may belong to neither of the two classes because of the unusual property of exhibiting dominance for mucoidity in two of these strains (42).

SECTION II. Results and Discussion

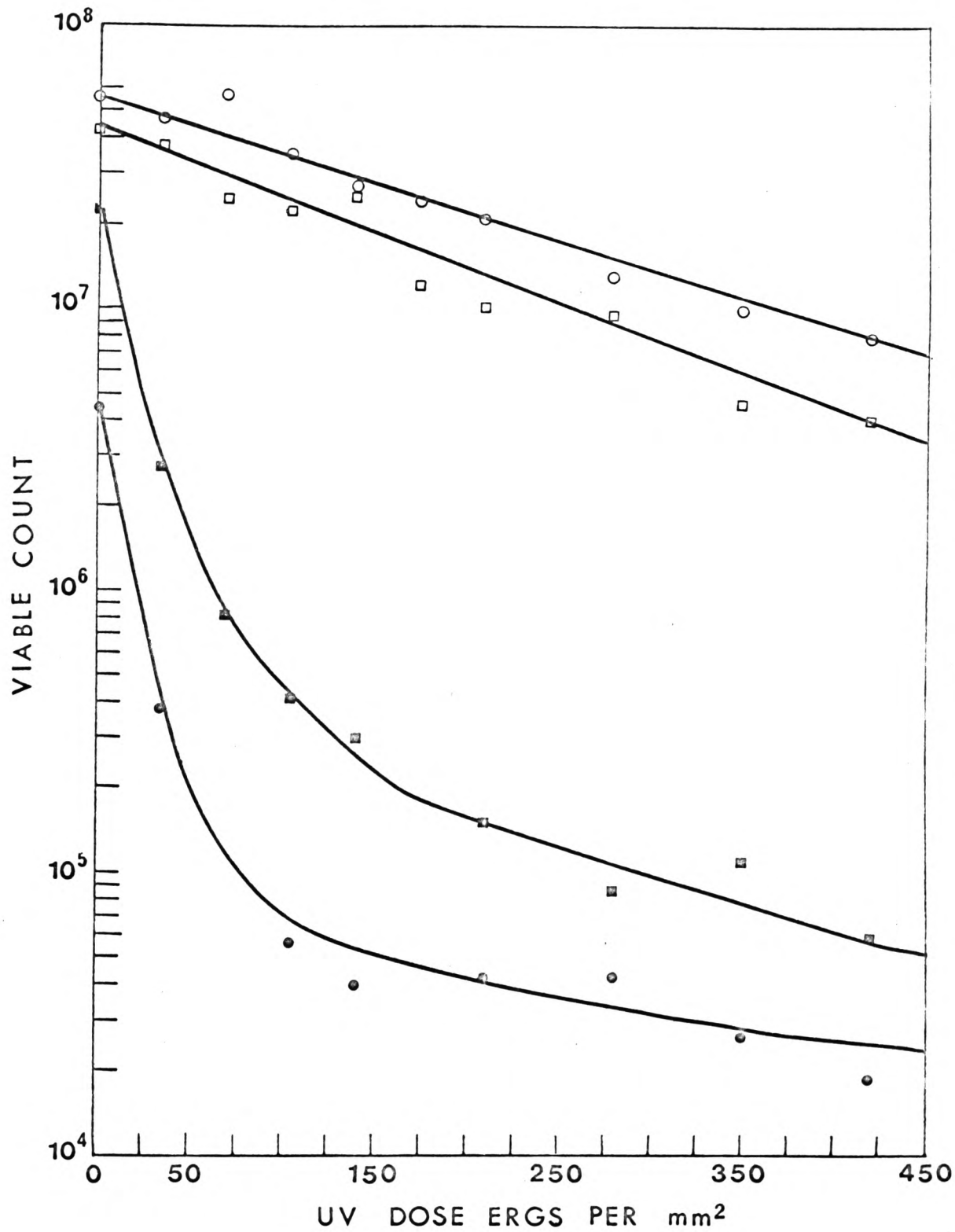
UV Survival Curves:- Lon strains have a biphasic UV killing curve (64, 75). The reason for this is presumed to be the presence in the culture of a resistant population of cells. This result was confirmed by following the survival of the lon strain E.coli A1899NM (Fig. IV). The curve shows a rapid decrease in the

number of survivors up to about 100 erg/mm^2 (approx. 2% survivors). The rate of death then decreases and for the range of doses used (up to 450 erg/mm^2) shows a logarithmic death rate with the number of survivors halving every 150 erg/mm^2 (LD_{50} = 150 erg/mm^2) The wild-type AB1157 lon$^+$, for doses between 0 and 450 erg/mm^2 shows a logarithmic death rate (LD_{50} = 125 erg/mm^2) with no shoulder, in agreement with earlier results (75) (Fig. IV).

With these two curves as references, the UV-killing curves of the two derived strains TG894 lon$^-$ and TG894LL lon$^+$were determined (see Table I) (Fig. IV). There is close similarity in the killing curves for the two lon$^-$ strains and also for the two lon$^+$ strains. TG894 lon$^-$ showed a rapid rate of killing up to 75 erg/mm^2 (2.5% survival) and then an exponential rate of killing (LD_{50} = 225 erg/mm^2). TG894LL lon$^+$ possesses an LD_{50} of 150 erg/mm^2, more in line with the reference strain than TG894. Still, the results for TG894 are close enough to those for AB1899NM to make any difference not significant. The two derived strains were, therefore, confirmed in their identities and could be used for studies on the effect of thymine starvation on lon cells.

DNA Synthesis after Thymine Starvation:- It has been previously reported that there is a correlation between the restoration of the DNA to mass ratio of lon$^+$ cells and the recommencement of cell division after thymine starvation (38). A slow rate of recovery of the DNA content of lon$^-$ cells could, therefore, lead to an increased delay or loss of the ability to divide. If the rate

Fig. IV

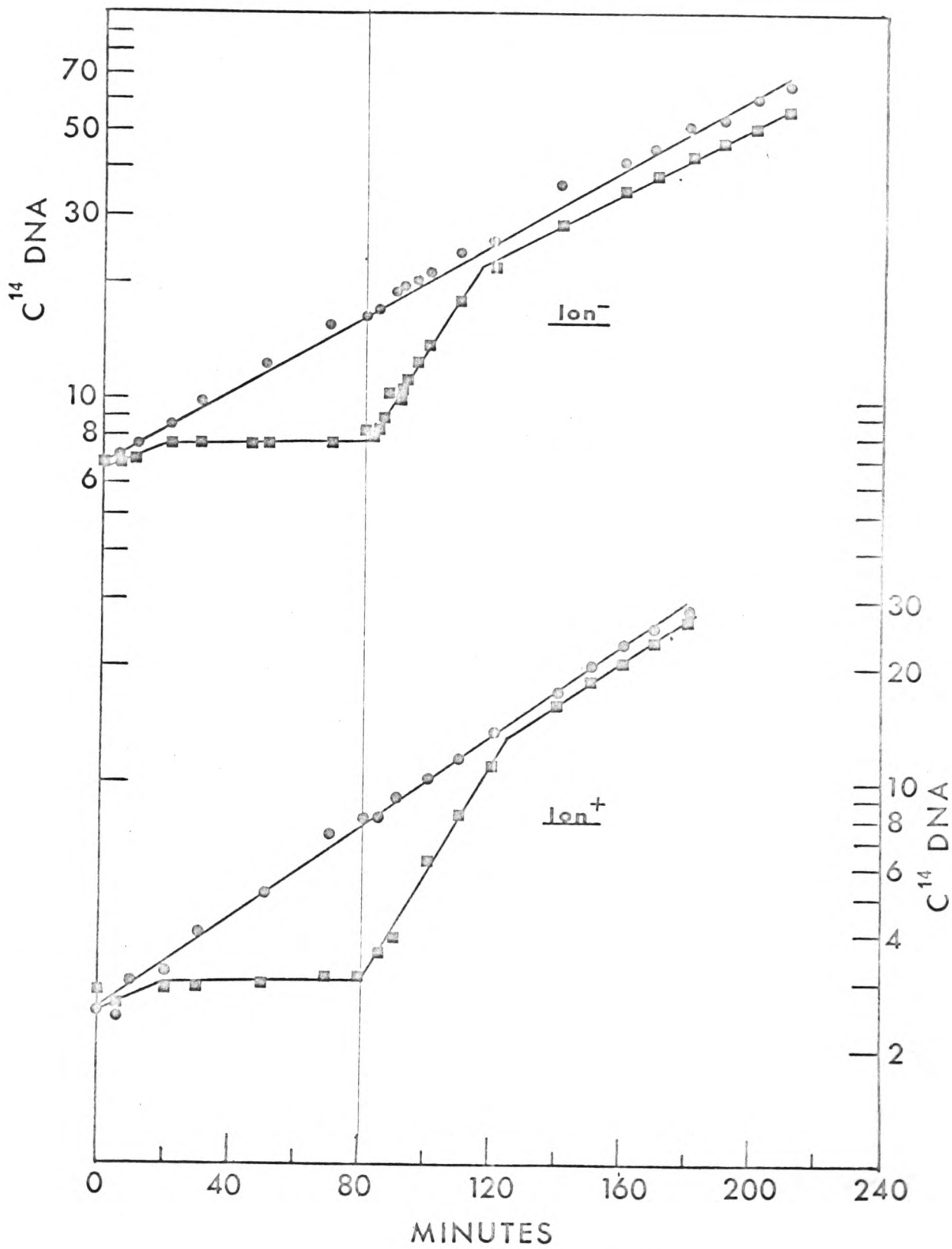


Viable count after UV irradiation. Cultures in the logarithmic phase of growth in minimal medium were irradiated. After irradiation, samples were pour-plated on L-agar containing added thymine (10 µg/ml) and incubated in the dark. AB1157 (lon^+), □; AB1899NM (lon^-), ■; TG894LL (lon^+), ○; and TG894 (lon^-), ●.

of DNA synthesis in the thymine starved culture is the same as or less than that in the control culture, the cells will never regain their DNA/mass ratio and may thus lose the ability to divide. I have, accordingly, compared the kinetics of DNA synthesis in TG894LL (lon$^+$) and TG894 (lon$^-$) cells after 80 min of thymine starvation*. The course of DNA synthesis is identical in the two strains (Fig. V). DNA synthesis continues at a decreased rate for about 20 min after the removal of thymine from the medium and then stops. As mentioned earlier, this would suggest that in this K12 strain, as in others (20), there is a pool of thymine in growing cells. DNA synthesis recommences as soon as thymine is reached, and continues faster than in the unstarved controls. Whereas the control culture doubles its DNA content in 50-60 min, during this period of rapid DNA synthesis, the DNA content of the starved culture doubles in 20-22 min. When the DNA of the starved cells is restored almost to the level in the control cultures, their rates of synthesis become essentially the same. These kinetics for DNA replication after thymine starvation resemble those for E.coli JG151 (35). The treated cells regained their normal complement of DNA in 35-45 min. The fact that the DNA content of the cell, and therefore the DNA/mass ratio, is restored indicates that it cannot be failure to regain this ratio which causes the inhibition of cell division in lon$^-$ cells.

* The mass doubling time of these cultures was about 60 min. Taking into account the 20 min thymine pool, in order to starve the cells for one mass doubling time, they had to be grown in thymineless medium for 80 min.

Fig. V

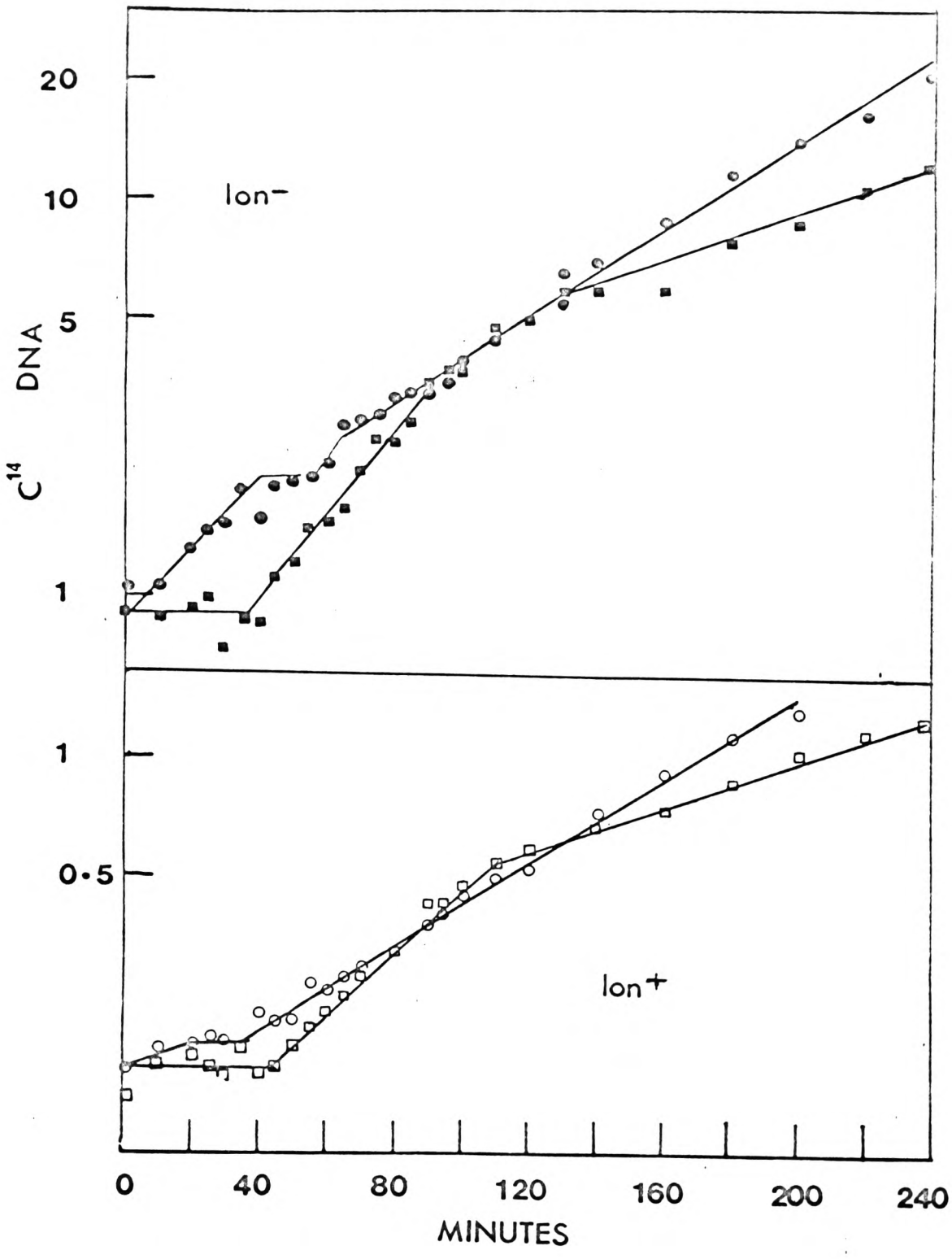


DNA synthesis in TG894LL (lon⁺) and TG894 (lon⁻) during and after thymine starvation. Cultures were grown for three mass doubling times in minimal medium containing ^{14}C-thymine. At 0 min, the culture was filtered, washed, and resupended in fresh minimal medium containing ^{14}C-thymine (●), and in the absence of thymine (■). At 80 min, ^{14}C-thymine was added back to the starved cultures.

DNA Synthesis after Ultraviolet Irradiation:- The effect of ultraviolet irradiation (UV) on DNA synthesis in *lon*$^+$ and *lon*$^-$ cells was also looked at. The results were slightly different depending on the growth medium used after irradiation. When cells were grown on minimal medium, irradiated at 320 erg/mm^2 and resuspended in minimal medium, after a delay in DNA synthesis of 35 min (*lon*$^-$) and 45 min (*lon*$^+$), there was rapid DNA synthesis until the irradiated culture contained the same amount of DNA as the untreated control. The rate of DNA synthesis then decreased to less than that of the control culture (Fig. VI). Except for the final decrease in rate of synthesis, the picture here closely resembles that due to thymine starvation (Fig. V).

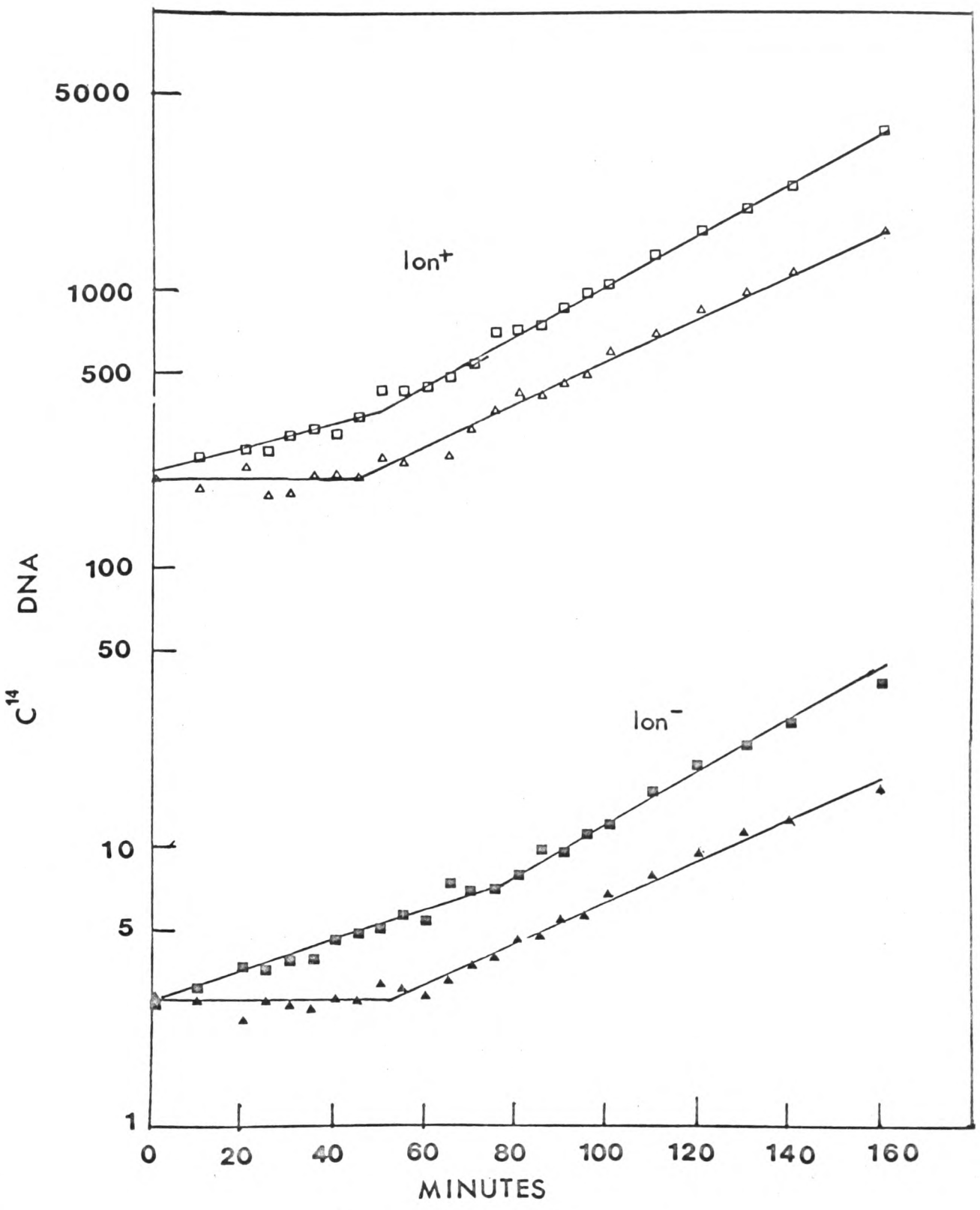
If the cells after irradiation at 480 erg/mm^2 were put instead into an enriched medium (minimal casamino acids), there was a delay in DNA synthesis of 45 min (the longer delay is presumably due to the higher dose of UV). DNA synthesis then recommenced, but at a rate slightly slower than the control culture (Fig. VII). This difference in the kinetics of DNA synthesis between these two sets of experiments is probably due to the post-irradiation medium. An experiment with a different *lon*$^-$ strain irradiated at 480 erg/mm^2 but resuspended after irradiation in minimal medium gave the same picture as cells irradiated with 320 erg/mm^2 and grown in minimal medium. This was only one experiment, but it is suggestive that after a "step-up" the DNA content of irradiated cells does not return to normal for at least some time. The difference would seem to be that after irradiation the doubling time of the DNA is 30-36 min

Fig. VI



DNA synthesis in TG894 (lon⁻) and TG894LL (lon⁺) after UV irradiation. Cultures were grown for three mass doubling times in minimal medium containing ^{14}C-thymine. At 0 min, the cultures were irradiated with 300 erg/mm^2 and diluted 1:5 into fresh medium containing ^{14}C-thymine. Unirradiated control cultures were run in parallel. Irradiated cultures, ■ , □ ; unirradiated cultures, ● , ○ .

Fig. VII



Treatment of these cells was the same as those in Fig. VI, except that irradiation was with 480 erg/mm and the cells were diluted 1:10 into minimal casamino acids medium, which is a 'step-up'. Again, unirradiated cultures were run in parallel. Irradiated cultures, △ , ▲ ; unirradiated cultures, □ , ■ .

irrespective of the medium of growth. Since the rate of movement of the replication point,is constant, if the cell reininitates after transfer to enriched medium so that the chromosome now replicates dichotomously, it will have the same rate of DNA synthesis as a cell grown in minimal medium which has been induced by UV to reinitiate. This means that the DNA to mass ratio of the irradiated cell will not catch up with that of the untreated cell for some time.

It was reported previously by Billen (14) that there is no increase in the rate of DNA synthesis after UV irradiation. My results show that there is a very marked increase in the rate initially. The difference between our results is likely to be due to the strain and the dose of UV used. Billen irradiated his cells with 600 erg/mm^2 which is almost twice the dose I used. This dose gave him a 24% survival of cells, a figure which can be explained only by the high UV resistance which he says is a property of E.coli 15T$^-$ (555-7). The damage caused to the DNA at this dose will remain the same and will be considerable. Using the results of Rupp and Howard-Flanders (128) that 45 erg/mm^2 results in 270 dimers/genome, the DNA of his irradiated cells will possess 3600 dimers/genome. (Billen estimates the chromosome to have 2100-3000 dimers.) At the dose I used (320 erg/mm^2), the damage would be 1900 dimers/genome using Rupp and Howard-Flanders' results. It seems likely that although the cells are able to repair the damage eventually, DNA replication is inhibited in Billen's cells even after the initial 20-30 min absence of uptake of radioactive thymine

(repair synthesis?) and recovery is slow.

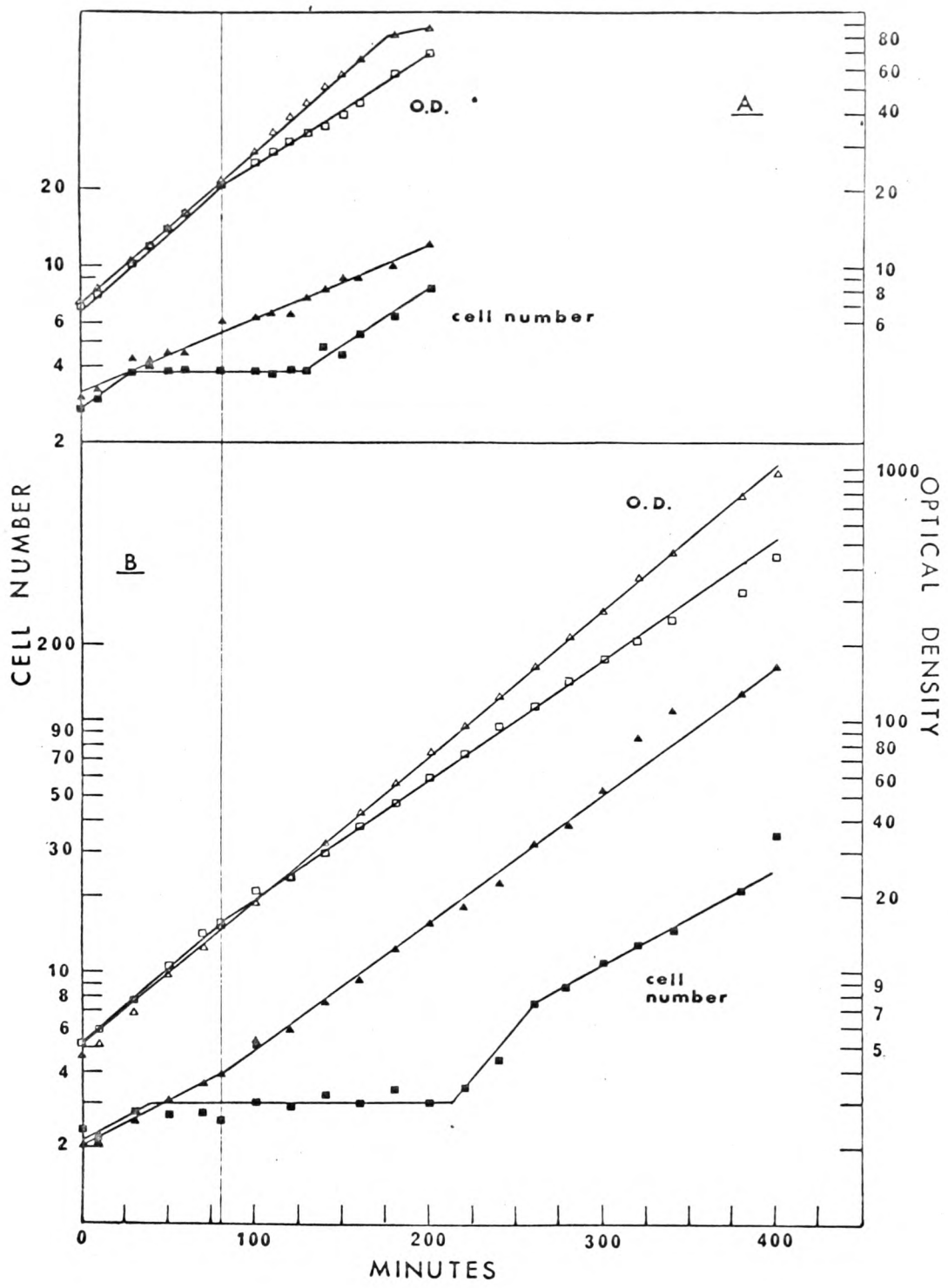
Cell Division after Thymine Starvation and UV Irradiation:-

It has been reported previously that lon-strains of E.coli lost the ability to divide after treatment with UV (5, 75), nalidixic acid (88), hydroxyurea (88), BUdR (165, 166), or X-rays (5). These treatments all act by inhibiting DNA synthesis. This suggested that probably thymine starvation would have the same effect. The advantage of this method of inhibiting DNA is that it is very easily controlled, its mechanism of action is known, and its action is specific for DNA.

All experiments were performed in minimal medium containing 10 µg/ml thymine. Log-phase cultures of the thymine requiring strains TG894LL (lon^+) and TG894 (lon^-) were filtered and resuspended in minimal medium in the absence of thymine (see Chapter 2). After 80 min of starvation, when the total mass (as measured by optical density) had increased about three fold, thymine was readded to the medium. Fig. VIII shows the results obtained for increase in cell mass (O.D.) and cell number.

The cells continued to divide for some time after the removal of thymine until the cell number had increased by 43-44%. The cell number then remained constant although optical density (O.D.) increased at the same rate as in the unstarved culture. On readdition of thymine to the lon^+ culture, there was a delay of 40-50 min before

Fig. VIII



Cell number in TG894LL (lon$^+$) and TG894 (lon$^-$) during and after thymine starvation. At 0 min, log-phase cultures growing in minimal medium were filtered, washed and resuspended in the presence (▲, △) and absence (■, □) of thymine (solid symbols, cell number; open symbols, optical density). At 80 min, thymine was added back to the starved cultures. B, To maintain the culture in logarithmic growth, the thymine starved culture was diluted into fresh warm medium at 80 and 250 min, and the control unstarved culture was similarly diluted at 80, 250 and 300 min. The graph has been corrected for these dilutions. A, lon$^+$; B, lon$^-$.

cell division recommenced. Cell division initially was at a faster rate than in the control cells. Although division was followed for only a short period, it may be assumed that once the cell number of the treated cultures approximates that of the control culture, their rates will be similar. It has been seen (Fig. V) that once the DNA content of the starved culture equals that of the unstarved culture, their rates of synthesis become the same. If the cells were to continue dividing at this rapid rate, they would either reduce the amount of DNA per cell or would divide off enucleate cells. Since this does not occur, it may be assumed that the rate of cell division returns to normal once most of the elongated cells have divided up. This has been found to be the case both in a $\underline{lon}^+$ and $\underline{lon}^-$ culture (166) and in JG151, a $15T^-$ strain (35, 38).

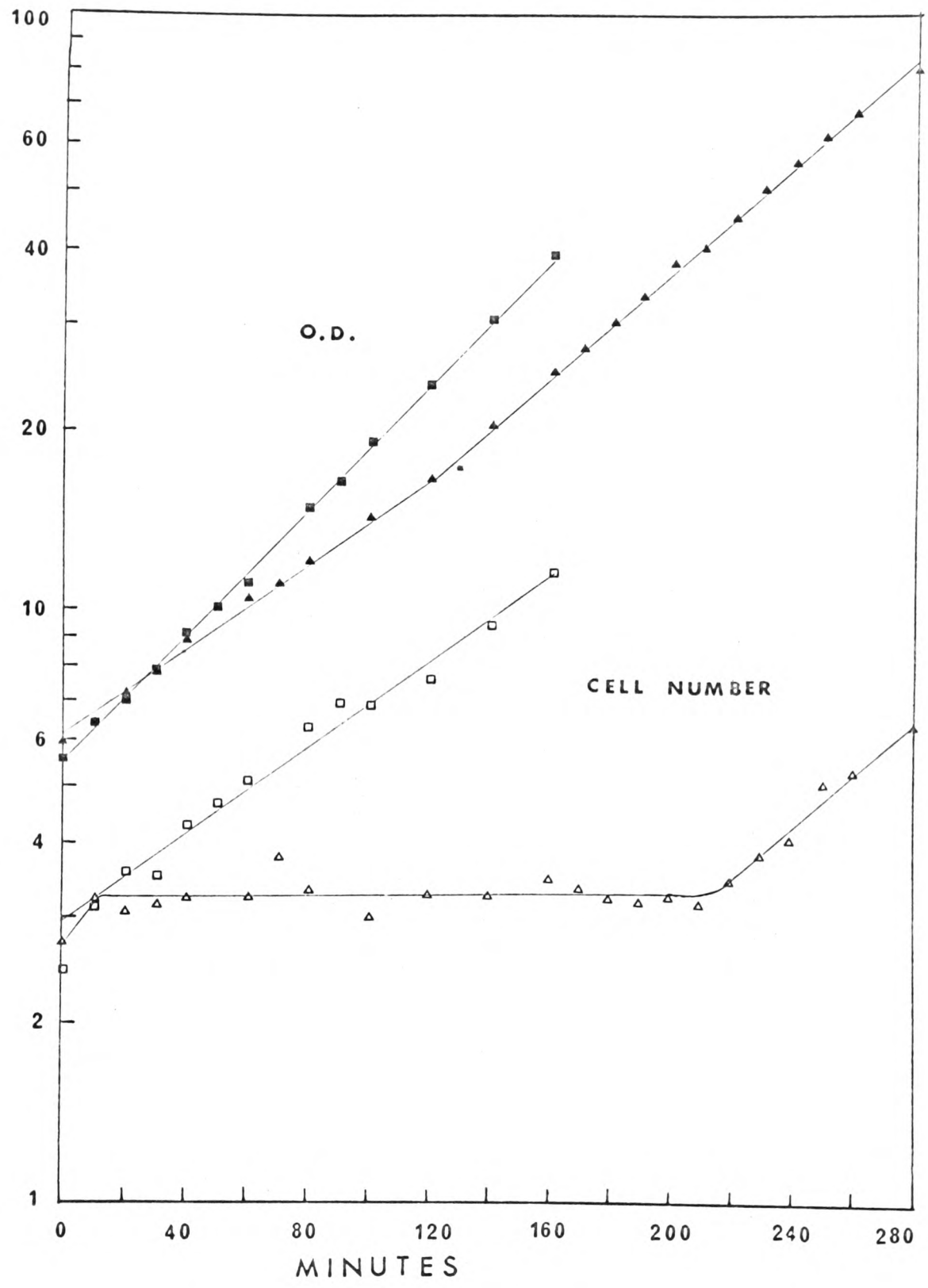
When thymine was readded to cultures of $\underline{lon}^-$ cells starved of thymine for 80 min, the delay before cell division recommenced was 120-140 min. Apart from this difference in the length of the delay period, the kinetics of division of the $\underline{lon}^-$ cell appears to be the same as for the $\underline{lon}^+$ cells. Cell division in $\underline{lon}$-filaments has been observed previously. However, in all cases, the cells were treated in some manner to induce division (1, 5, 8, 9, 51, 121, 144, 160, 175). This is the first inducation that $\underline{lon}$-filaments can divide under normal growth conditions. The reason why this was not observed earlier rests on the method used to study $\underline{lon}$. Previous work has involved plating UV or X-irradiated cells (this work is the first to use thymine starvation to induce filamentation), and

examining the plates the following day for colonies (viable cell count). However, it has been shown that plating can 'kill' cells which have been thymine starved (37). It seems likely, therefore, that previous workers have been enhancing the lon- effect by superimposing a plating death. Walker & Smith (166) subsequently also found that if lon⁻ cells were kept in liquid medium after a brief period of thymine starvation, the filaments recovered completely.

It was possible that thymine starvation is a milder treatment for the cell than UV-irradiation, and that after UV, the cells are unable to recover as has been reported previously. A lon⁻ culture was irradiated with 320 erg/mm^2 of UV-light and growth and cell number followed. Fig. IX shows that the cells can recover from UV-irradiation and the delay in division is 210 min. From Fig VI, it may be seen that this dose of irradiation inhibits DNA synthesis for 40 min which means that division is delayed an extra 170 min. This figure is considerably in excess of the 110 min found for a similar period of thymine starvation. It is concluded that this dose of irradiation is high enough to cause damage to the cell which inhibits cell division in excess to the delay due to inhibition of DNA synthesis. This result confirms a previous report that E.coli B regained the ability to divide after UV-irradiation (31, 154).

It has been previously reported (35) that the length of the delay between readdition of thymine and recommencement of division

Fig. IX



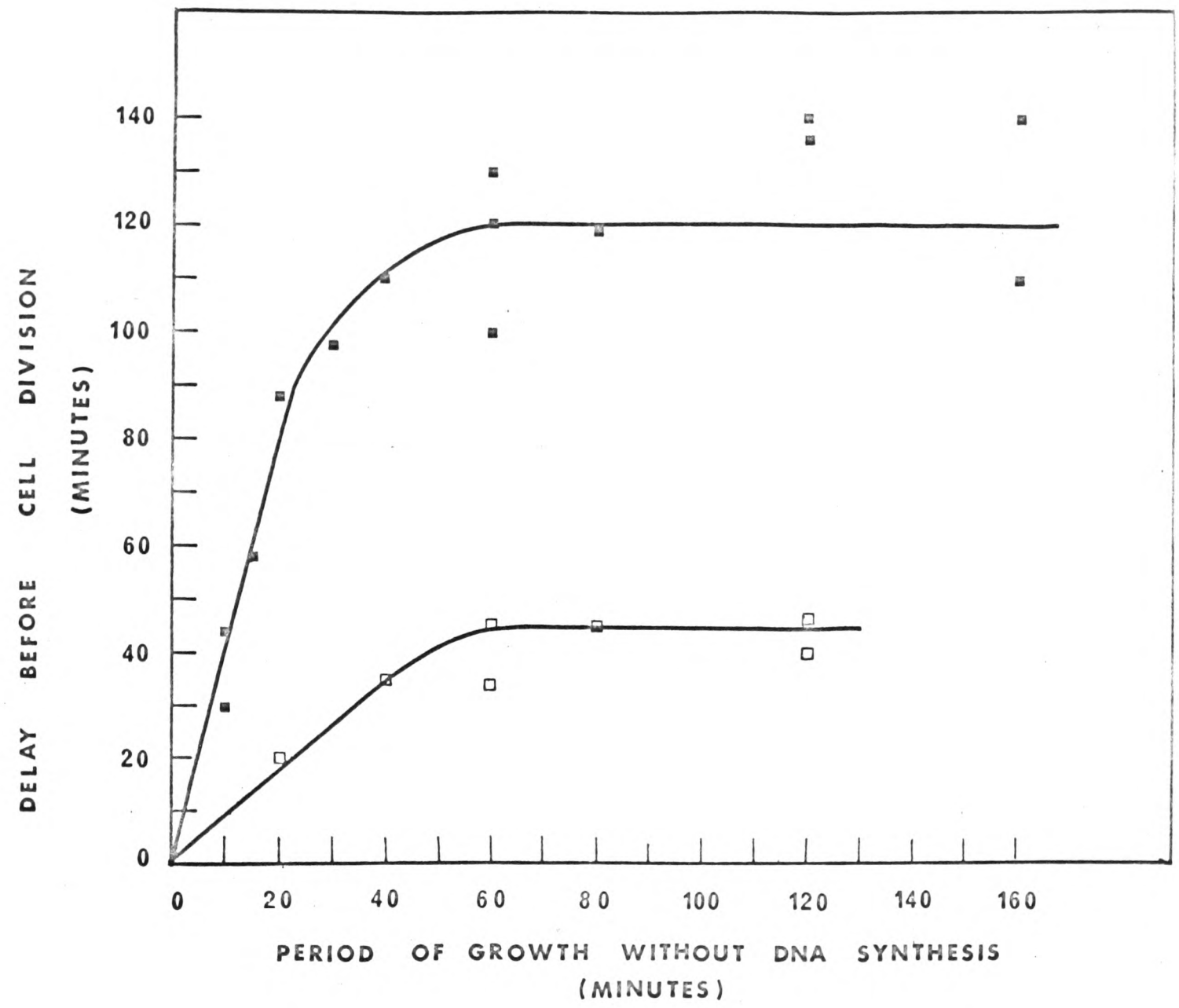
Cell division in a lon⁻ culture after UV irradiation. A culture of AB1899NM (lon⁻) was grown for 3 mass doubling times in minimal medium. The cells were then irradiated with 320 erg/mm^2 and diluted 1:4 into fresh medium. A control unirradiated culture was run in parallel. Irradiated culture, ▲ , △ ;; unirradiated culture, ■ , □ .

is a function of the length of the preceding period of growth without thymine. Using long periods of thymine starvation, TG894LL showed cell lysis, presumably due to induction of a lysogenic phage (probably P1). For these and subsequent experiments, therefore, the *lon*$^+$ strain AB2497 was used. Since all the *lon*$^+$ and *lon*$^-$ strains used are derived from AB1157, they should all have the same genotype except for the locus for thymine requirement and the *lac* gene of TG894LL.

AB2497 (*lon*$^+$) was starved of thymine for periods varying from 20 to 120 min, and the delay between readdition of thymine and the beginning of cell division measured. Up to 60 min of thymine starvation, the delay increased linearly to 45 min. Longer starvation periods did not increase the delay period any further (Fig. X). TG894 (*lon*$^-$) was similarly starved of thymine for periods varying from 10 to 160 min. Again there was a linear increase in the delay period with a maximum at 60 min of starvation. The maximum delay in this case was 120-140 min. (Fig. X). This means that after the same length of time of thymine starvation, *lon*$^-$ cells show about three times the delay in cell division as *lon*$^+$ cells.

Donachie (35) obtained similar results to those for the *lon*$^+$ strain with two other strains of *E.coli*, JG151 and B/r/1 T$^-$. Although he has shown a maximum delay of 60 min after 80 min thymine starvation, his results can be redrawn with the graph showing a maximum delay of 45-50 min obtained after 60 min thymine starvation.

Fig. X



Relationship between pregrowth in the absence of DNA synthesis (thymine starvation) and the delay before cell division after DNA recurs (readdition of thymine). To allow for the thymine pool, the length of time of no DNA synthesis is taken as the time of thymine starvation less 20 min. TG894 (*lon*$^-$), ■ ; AB2497 (*lon*$^+$), □ .

Since JG151 may be presumed to be lon$^+$ and B/r/1 T$^-$ is lon$^-$ rad$^-$ (40), this fits the results for AB2497 perfectly.

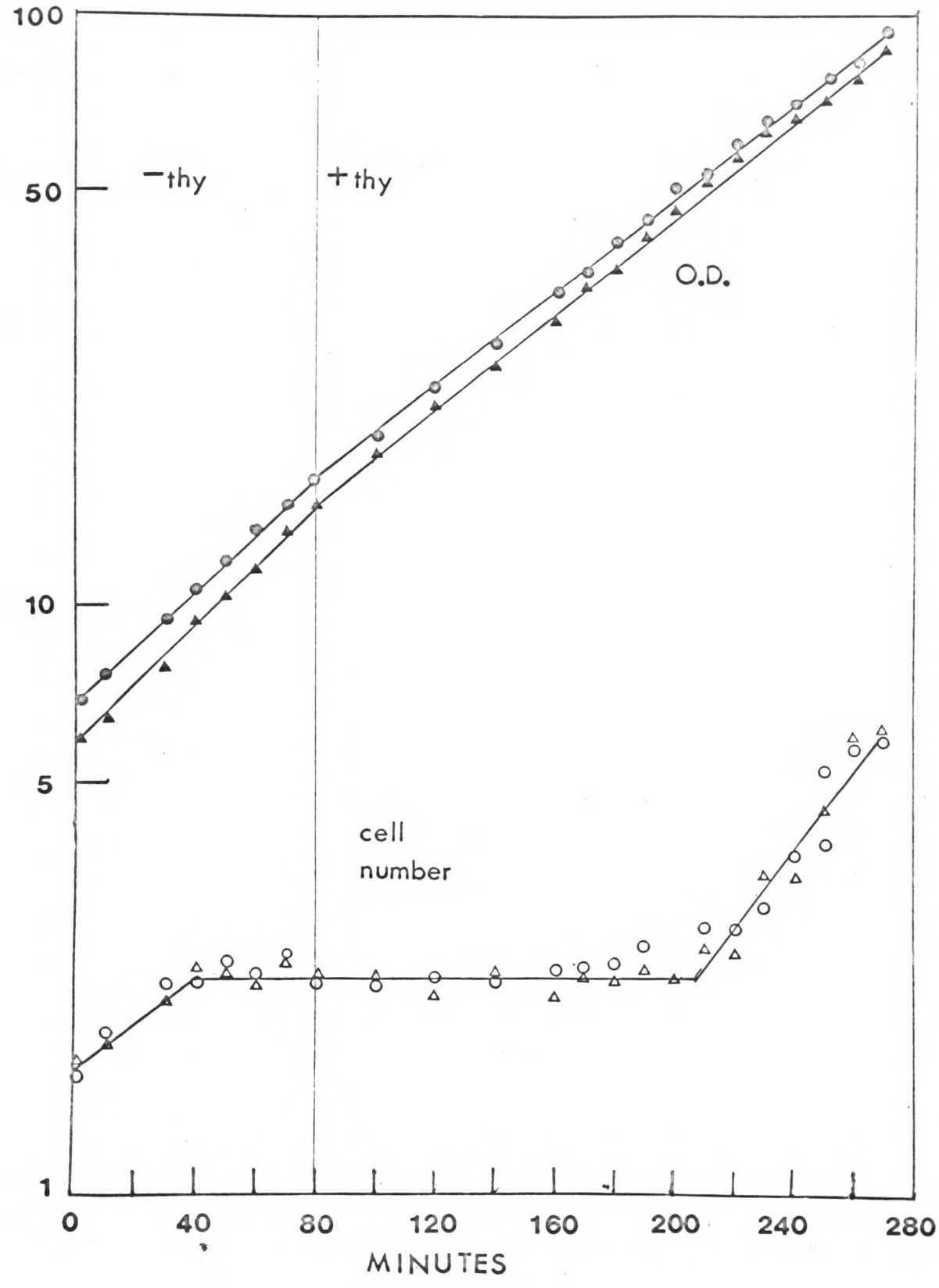
This 120 min delay can be seen in many ways. It could be two mass doubling times or 2-3 round of DNA replication (depending on the length of C in the cells). It now appears likely that at 10 µg/ml thymine, the C time of these cells was slightly increased (117). However, the same delay in division after thymine starvation was obtained as with cells grown in the presence of 30 µg/ml thymine (see below) (Fig. VIII and XI). This suggests that the period of delay in division is related to cell growth rather than DNA replication.

Effect of Thymine Concentration on Delay in Cell Division:-

As mentioned earlier, Pritchard and Zaritsky (117) found that thymine-requiring E.coli cells growing in the presence of less than 10 µg/ml thymine show an increased time for replication of the chromosome. Since these experiments were performed in the presence of 10 µg/ml thymine, it was conceivable that the results were affected by the cells being very slightly thymine-starved.

Consequently, cell division was followed in lon$^-$ cells after thymine starvation of two parallel cultures, one containing 10 µg/ml thymine and the other 30 µg/ml. The two cultures followed identical patterns (Fig. XI) and, as in other experiments, after 80 min growth in the absence of thymine (60 min starvation) showed a delay in division of about 120 min. The only notable difference between

Fig. XI



Effect of thymine concentration on division of lon⁻ cells (TG894) after thymine starvation. Cells were treated as in Fig. VIII B except that before and after thymine starvation, cells were grown in the presence of 10 µg/ml (●, ○) or 30 µg/ml (▲, △) thymine.

the two cultures is that the cells grow in the presence of 30 µg/ml thymine were slightly smaller than those grown at 10 µg/ml. This is shown by the fact that although the cell numbers are the same, the high-thymine culture shows a lower optical density. This is what would be expected if the cells growing in 10 µg/ml. This is shown by the fact that although the cell numbers are the same, the high-thymine culture shows a lower optical density. This is what would be expected if the cells growing in 10 µg/ml take longer to complete a round of replication. In this case, the chromosomes would be multiforked since the mass for initiation (M_i) (34) would be reached before the previous round was completed. Therefore, at the time of division, each cell would be larger than one 'unit cell' long (36).

These results indicate that the results obtained for cell division after thymine starvation in lon$^-$ cells is not affected by the concentration of thymine used.

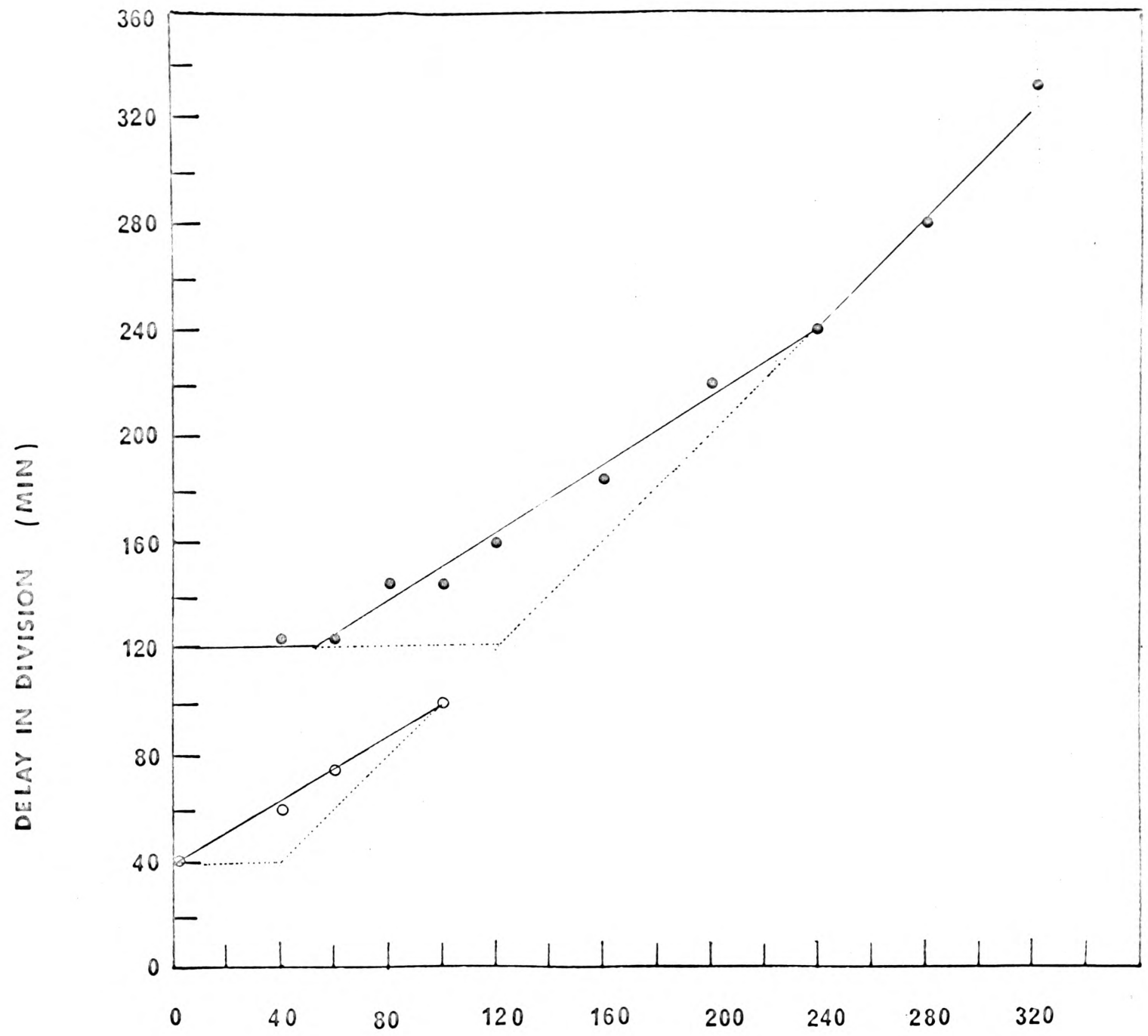
Amino Acid Starvation of Thymine Starved Cells:- When thymine is readded to thymine starved lon$^-$ cells, there is a delay before division occurs. It is possible that this delay is the time required to synthesize a protein necessary for division which is inactivated or not produced during thymine starvation. If this were the case, amino acid starvation during this time would delay recovery of the ability to divide by a time equal to the period of amino acid starvation.

After growing the cells for 80-85 min in the absence of thymine, the culture was filtered and resuspended in medium containing thymine but no amino acids. After varying lengths of time, amino acids were readded to the medium and cell growth (optical density) and cell number followed. Fig. XII shows a plot of the total delay in cell division versus the time of amino acid starvation. After 80 min thymine starvation, a delay of 120 min before division is expected even in the presence of amino acids. Amino acid starvation for times up to 50 min does not increase this period. For longer times of amino acid starvation, division is delayed further but the extra delay period is less than the period of amino acid starvation. For this reason, with increasing lengths of amino acid starvation, the extra delay over and above that expected (120 min + time of amino acid starvation) decreases until at 240 min, division occurs immediately on readdition of the amino acids.

If *lon*$^+$ cells are treated similarly, they show a slightly different pattern. They show a maximum delay in division after readdition of amino acids if the amino acids are readded at time zero. This delay then decreases linearly until after 100 min of amino acid starvation, division occurs immediately on readdition of amino acids.

These results present two points. They indicate another facet of the difference between *lon*$^+$ and *lon*$^-$ cells, and they provide a further clue to the mechanism of cell division in *E.coli*. Although protein synthesis is necessary for cell separation to occur, cell

Fig. XII



Cell division after thymine starvation followed by amino acid starvation. Cells were thymine starved for 60 min, filtered, and resuspended in the presence of thymine and the absence of amino acids. After various times, amino acids were readded and the time after readdition of thymine when cell number started to increase was noted. AB2497 (*lon*$^+$), ○; TG894 (*lon*$^-$), ●. The dotted lines represent the delay in division expected if amino acid starvation causes no increase in the delay beyond the time of amino acid starvation itself.

division up to this stage can be completed at a slower rate in the absence of protein synthesis. It is apparent that the sites for cell division must be either determined even in the absence of chromosome replication, or no protein synthesis is required to form the division site and it is determined by some other method e.g. the site of attachment of DNA. Donachie & Begg (unpublished results) found that the former is the case.

Although lon$^+$ and lon$^-$ cultures recover their DNA content at the same time (Fig. V), the sequence of events resulting in division (which I will call the division process) in the absence of amino acids requires a different length of time. In lon$^+$ cells, the requirement for protein synthesis in the division process begins decreasing immediately: After 100 min of amino acid starvation, no major protein synthesis is required for division to be completed. Bird and Lark (178) reported that the time taken for the replication fork to traverse the E.coli chromosome varied with the growth rate. In aspartate medium (doubling time 120 min), the 15T$^-$ chromosome took 80 min to replicate itself. It seems not unlikely that during amino acid starvation, the time for completion of a round of DNA synthesis is similarly slowed. The 100 min delay in the ability to divide would thus be the time for termination to occur. In this case, if amino acids are restored to the medium after 40 min, the rate of DNA synthesis would be expected to increase and, assuming a normal time for replication of the chromosome of 40 min, the time before completion of the round would be 40 + (60/100 x 40) = 64 min. I found the time experimentally to be 60 min.

Similarly, after 60 min amino acid starvation, the expected delay is 60 + (40/100 x 40) = 76 min compared to 75 min by my results.

If these calculations are applied to the results for lon$^-$ cells, a different finding emerges. In this case, the replication time, which would be required to give the observed delays before division is 87 min, so that after 160 min of amino acid starvation, 160 + (80/240 x 87) gives 189 min for the time of completion of the round of DNA synthesis. The results show a time of 188 min. Again division occurs when the round of DNA replication is completed. This suggests that lon$^-$ cells possess a C time approximately twice as long as that of lon$^+$ cells under the growth conditions normally used. During amino acid starvation, each has its C time increased about 2.5 fold.

Whereas for lon$^+$ cells the relationship given above holds all the way back to the origin, this is not the case with lon$^-$ cells. For the first 50 min of amino acid starvation, the delay before cell division remains constant (120 min). This 120 min may be the time required for decay of an inhibitor of cell division. This is suggested by the fact that after 50 min amino acid starvation, the expected delay before division is 50 + (190/240 x 87) = 119 min.

In summary, in lon$^-$ cells there is a process (decay of an inhibitor of division) which takes 120 min to complete, in the presence or absence of amino acids, before division can occur. Division can also not occur before the chromosome has terminated.

Whereas in the presence of amino acids termination occurs before the 120 min process is completed, amino acid starvation slows the rate of DNA synthesis so that the time for termination may exceed 120 min. After 50 min amino acid starvation, the time for termination is 120 min and for longer periods of starvation the time for termination is longer.

Cell Division after Penicillin: Thymine starvation results in a cessation of DNA synthesis. Inhibition of cell division is a secondary process subsequent to this. The *lon*-induced filamentation could be due to inhibition of DNA synthesis or it could be due to the subsequent inhibition of the cell division process itself.

Penicillin interference with the transpeptidation which links the penultimate D-alanine of one of peptide chain to the free amino group of the neighbouring chain. The result is a weakened cell wall lacking cross-linking of its chains of mucopeptide (173). At high concentrations, this rapidly kills the cells, however, at very low centrations (1.5 - 50 u/ml) cell division is inhibited without affecting growth (151). DNA, RNA and protein synthesis continue normally. So, at low concentrations of penicillin, the cells grow into long filaments. When the penicillin is removed (e.g. by the addition of penicillinase) cell division resumes in wild-type cells (151).

To test the hypothesis that it is the inhibition of the division process rather than DNA synthesis which causes the *lon*

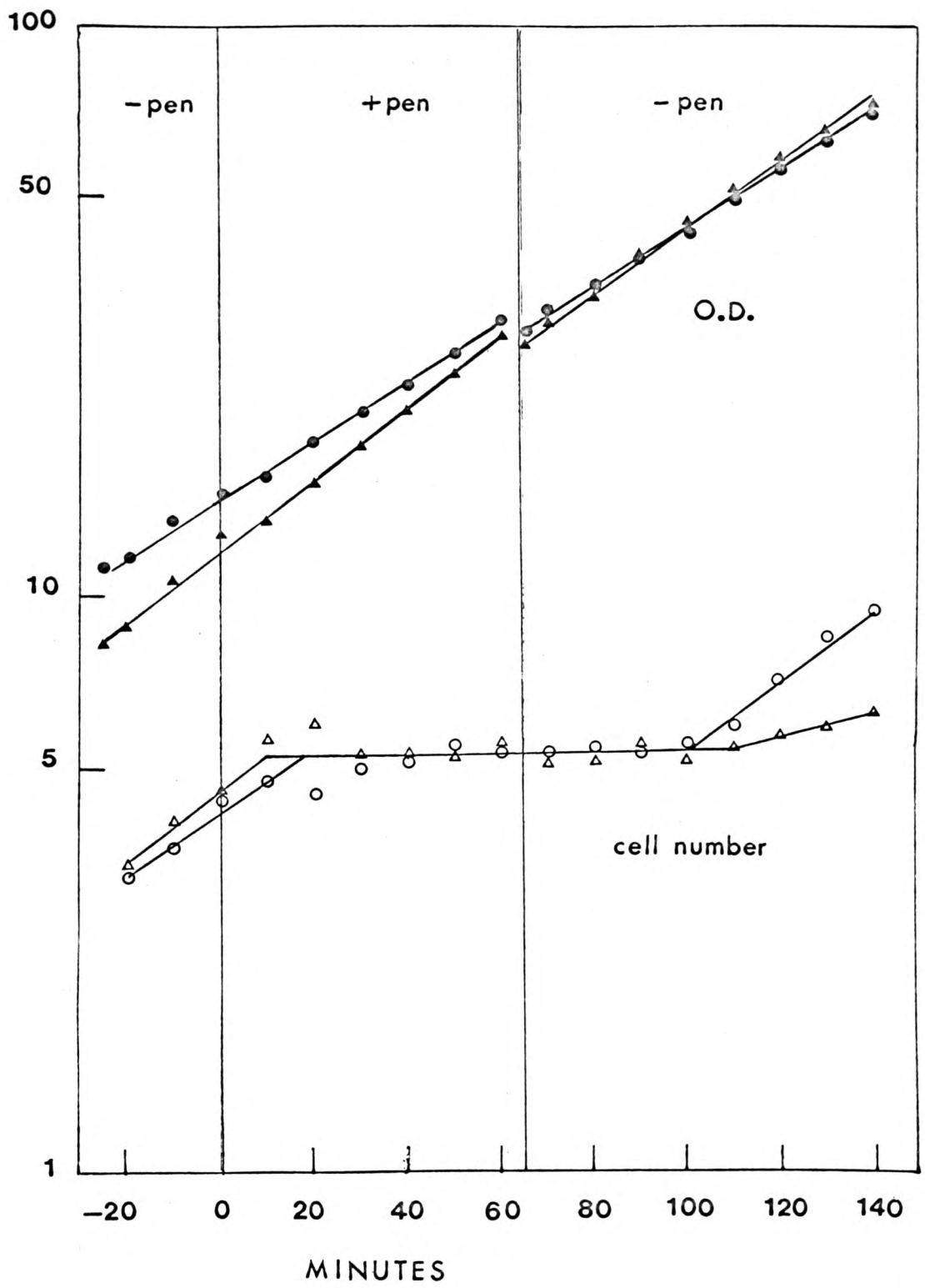
effect, cell division only was inhibited with low concentrations of penicillin.

To determine the minimal concentration of penicillin which would inhibit cell division without affecting growth, cultures of TG894 (lon$^-$) and TG894LL (lon$^+$) were grown to log phase in minimal medium and various concentrations of penicillin added. The optical density and cell number were followed. The minimal concentration of penicillin which could be used was 20-25 u/ml.

In the presence of this concentration of the antibiotic, both the lon$^+$ and lon$^-$ organisms responded similarly (Fig. XIII). Cell division stopped within 20 min whereas growth rate was unaffected. After 65 min, penicillinase was added to give a concentration of 1.3µg/ml. Growth rate was unaffected, but after 37 min (lon$^-$) or 45 min (lon$^+$) division restarted.

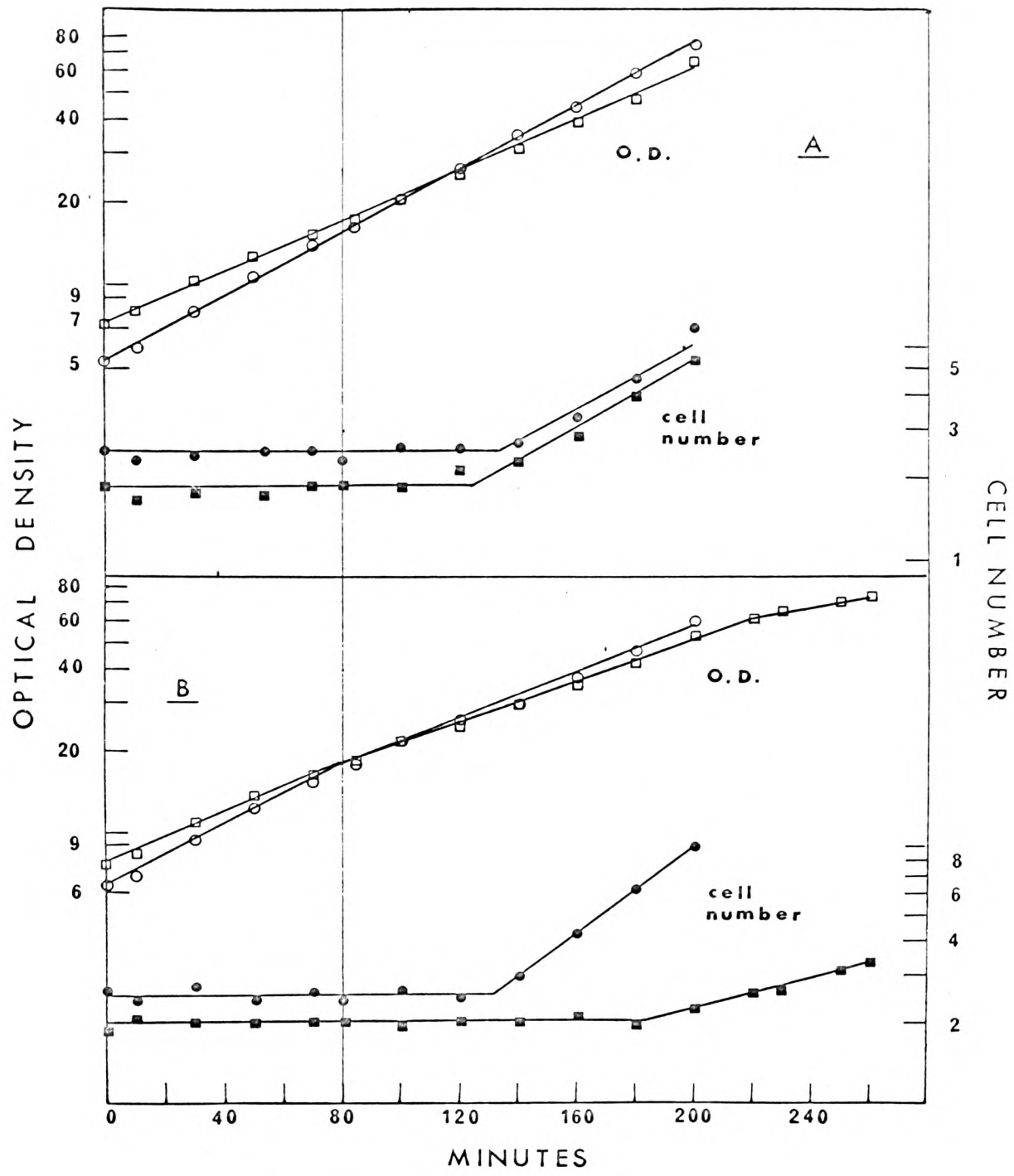
Fig XIV represents two experiments performed simultaneously on two halves of the same culture. Minimal grown log phase cultures of TG894 and TG894LL were filtered and resuspended in the presence (Fig XIV A) or absence (Fig XIV B) of thymine. Penicillin was added to all four cultures to a final concentration of 25 u/ml. Fig XIV A shows results similar to those of Fig XIII except that cell division in this case stopped immediately in both lon$^+$ and lon$^-$ cultures. Penicillinase (0.3 µg/ml) was added after 80 min and division restarted 44 min (lon$^-$) and 52 min (lon$^+$) after this.

Fig. XIII



Effect of low concentration of penicillin on cell division. Penicillin (20 u/ml) was added at time 0 to log-phase cultures in minimal medium. After 65 min, penicillinase (1.3 µg/ml) was added to the culture. Cell growth (O.D.) and cell number were followed. TG894LL (lon$^+$), ▲, △; TG894 (lon$^-$), ●, ○.

Fig. XIV



Effect of penicillin (A) and penicillin with thymine starvation (B) on cell division in TG894 (lon⁻; ■, □) and TG894LL (lon ; ●, ○); solid symbols = cell number, open symbols = optical density. At 0 min, penicillin (15 μg/ml) was added to the cultures (A), or the cultures were filtered, washed and resuspended in minimal medium containing penicillin (15 μg/ml) in the absence of thymine (B). At 80 min, penicillinase (0.3 μg/ml; A) or pencillinase (0.3 μg/ml) with thymine (10 μg/ml; B) was added to the cultures.

In Fig X B, which at 0 min had thymine removed from the medium and penicillin added, again division ceased immediately. At 80 min, thymine was added to a concentration of 10 µg/ml as well as penicillinase (0.3 µg/ml). The lon$^+$ culture divided at exactly the same time as the culture not starved of thymine. The lon$^-$ culture divided 100 min after the addition of thymine and penicillinase. These results indicate that penicillin and thymine starvation act at different points on the division process and penicillin treatment does not mimic thymine starvation.

The effects of thymine starvation and penicillin treatment are not additive either. With lon$^+$ cells, cell division occurred at exactly the same time irrespective of whether they were thymine starved during penicillin treatment or not. With the lon$^-$ cells, the effect seen is simply that of the thymine starvation and cell division is delayed 100 min from the addition of thymine and penicillinase.

It was noticed during the experiments with variable length of thymine starvation that the longer lon$^-$ cells were starved, the fewer cells seemed to recover. This was explicable by saying that as the cells become larger, they have less chance of recovery from thymine starvation. To check this, TG894 (lon$^-$) cells were treated with penicillin before thymine starvation to cause them to filament slightly. The results are listed in Table II. On the whole, the results are much what might be expected with cell division being delayed a similar length of time to cells which had

been thymine starved only. One exception is the very short delay found after 80 min of penicillin followed by 20 min of thymine starvation. This must be just assumed to be an anomalous point. The other discrepancies in Table II are the results for delay in division after penicillin treatment for 120 min followed by 60 min of thymine starvation. In this case, the cells did not divide at all in the time studied, so that the cells have either lost the ability to divide or it is very much delayed. I believe the former is true and the cells are killed by such a long period of inhibition of cell division. It is unlikely that the cells were killed by the penicillin treatment, since Stárka & Moravová (151) treated E.coli B with penicillin for 150 min with no harm to the cells. Therefore, it seems the cells have lost the ability to divide (and will die) due to the two treatments which resulted in 200 min of combined treatment (120 min penicillin + 60 min actual thymine starvation + 20 min using up the thymine pool*). Considering that there is a delay of 120 min expected due to the thymine starvation, this brings the total to 320 min. (During this time, the cell mass (O.D.) increased about $5\frac{1}{2}$ fold so that the cells are 32-64 times the normal cell length.)

Effect of pantoyl lactone:- There have been several reports that pantoyl lactone stimulates recovery in filaments of lon-strains (5, 40, 160). However, apart from the original work of Grula and Grula (63), no report of the mechanism of action of pantoyl lactone, especially on lon-cells, has been published. It is already

*There is no division during depletion of the thymine pool since it occurs during the delay in division subsequent to penicillin treatment.

TABLE II

Penicillin[a]	Thymine[b] Starvation	Delay[c] Found	Delay[d] Expected
40	60	100	120
	100	140	120
80	20	48	80
	60	110, 160	120
120	60	>220,>160	120

a length of time of penicillin treatment (minutes)

b length of time of thymine starvation (minutes)

c Delay between addition of thymine to starved culture and recommencement of division (minutes). As in Fig. V the cells appear to have a 20 min thymine pool so that starvation time is taken as 20 min less than the period of time in thymine-free medium.

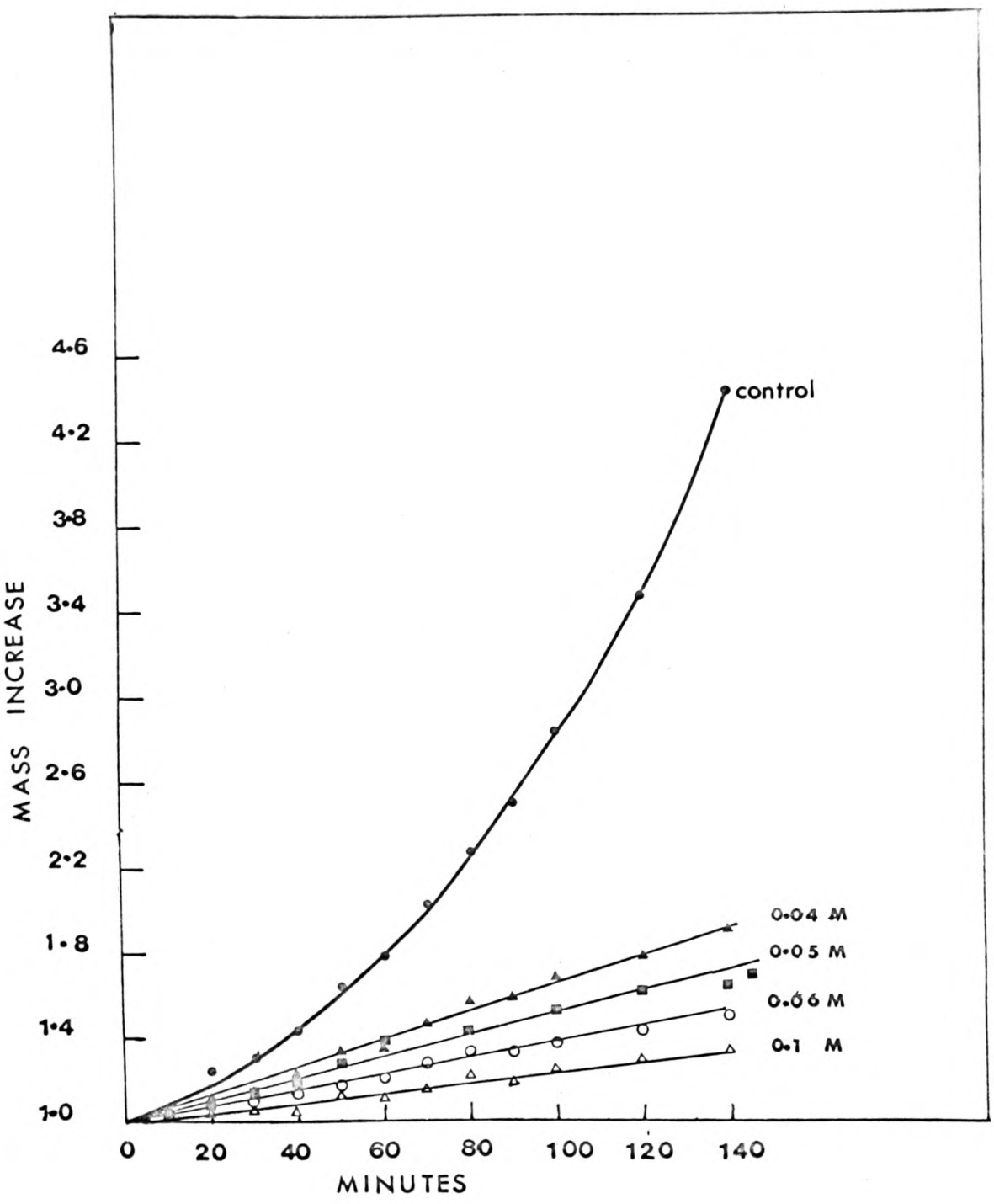
d delay expected from Fig X if cells are thymine starved only (minutes).

known that inhibition of growth promotes the recovery of filaments. This possibility had to be ruled out if the reversal of filamentation was to be significant.

Adler (5) and van de Putte (160) used pantoyl lactone at a concentration of 0.08M to induce division in lon-filaments. Donch (40) used 0.75% pantoyl lactone (0.057M) to increase the survival of E.coli B after UV irradiation. In their work on division in Erwinia, Grula and Grula (63) used this compound at a concentration of 0.046M. Therefore, in my experiments, I have used concentrations of 0.04M, 0.05M, 0.06M and 0.1M. The results are seen in Fig XV. These results are, with one exception, all from a single experiment on different fractions of one culture of AB1899NM (lon$^-$). The result for0.05M pantoyl lactone is from an earlier experiment with TG991 (lon$^-$).

There is a positive correlation between the concentration of pantoyl lactone and inhibition of growth. Even at 0.025M pantoyl lactone (not shown on graph), there was slight inhibition of growth. Whereas the control culture increased at a logarithmic rate (mass doubling time = 70 min), cultures containing 0.04M - 0.1M pantoyl lactone show a linear rate of mass increase. This may have two explanations. It may be that only one of the two daughter cells on division continues growing. It is also possible that all cells grow, but at a very slow rate, and the linear plot is, in reality, the initial part of a slow logarithmic rate of growth. Over less than a doubling, the points of a logarithmic curve are difficult to distinguish from a straight line. Since

Fig. XV



Effect of pantoyl lactone on growth. Pantoyl lactone at various concentrations was added to cells in the log-phase of growth in minimal medium. Optical density was followed as a measure of increase in cell mass. Except for 0.05M, all results are from one experiment with AB1899NM, lon⁻. The result for growth in the presence of 0.05M pantoyl lactone was obtained in a separate experiment with TG991, a thy⁻ derivative of AB1899NM derived at the same time as TG894.

none of the treated cultures was followed for greater than a 1.9 fold increase in mass, it is possible that the cultures are growing logarithmically but at a slower rate than the control cultures. It therefore appears likely that pantoyl lactone reverses filamentation in *lon*$^-$ cells at least partially by slowing growth. Adler (pers. commun.) claims that he found that at suboptimal concentrations, there was no detectable lag or slowing of growth rate. Without seeing his data, no explanation can be offered, but even at a concentration as low as 0.025M, I found a slight slowing of the growth rate of *lon*$^-$ cells. Pantoyl lactone does appear to stimulate septum formation (152) but Grula and Grula in their original study (63) realized that although pantoyl lactone stimulated cell division after inhibition by various chemical agents, there seemed to be no reason why it should be able to correct the damage in all cases due to the diversity of the agents, and they left open their conclusions concerning the function of pantoyl lactone in normal division.

Effect of F-Medium: All the methods described for specifically inducing *lon*$^-$ cells to filament have involved inhibition of DNA synthesis or a decrease in the DNA/mass ratio by some other means e.g. a step-up. It has been reported, however (170, 171), that *E.coli* B, when grown in a broth enriched with NaCl, lysine and casamino acids (F-medium) gave branched filaments. This was not found with *E.coli* E26. These results suggested that the reason for the different reactions of these two strains might rest in the *lon* gene. *Lon*$^+$ and *lon*$^-$ strains were grown overnight in

nutrient broth. 0.5 ml of this culture was filtered to remove most of the culture medium and the cells added to 10 ml of F-medium. Growth of the cells was observed periodically. The lon$^+$ cells were found to increase slightly in size and then to continue dividing, whereas the lon$^-$ culture contained filaments which increased in length with increasing time in F-medium. Fig XVI shows lon$^+$ and lon$^-$ cells on transfer to F-medium and 180, 240 and 300 min later. The lon$^-$ culture consists largely of filaments after 3 hrs growth in this medium, whereas the lon$^+$ culture appears fairly normal although the cells are somewhat enlarged. If part of the filamentous lon$^-$ culture was filtered and resuspended in nutrient broth, many of the filaments divided off normal sized cells which, within a couple of hours predominated in the culture. If growth in F-medium was allowed to continue, the lon$^+$ cells often formed spheroplasts, as did many of the non-filamentous lon$^-$ cells. Attempts were made to see if these spheroplasts could recover, but they could not be recovered by filtration through a Millipore filter containing 0.22μ or 0.45μ pores. The tendency of lon$^+$ cells to form clumps and the sphero plast formation were both suggestive of a surface change of the organisms.

Walker and Smith (166) reported that if lon$^-$ cells were shifted up from minimal medium to yeast extract-tryptone broth or glucose-casamino acids-adenosine-uridine medium, there was a temporary inhibition of cell division. It was considered possible that this was the effect observed with F-medium. If optical

Growth of <u>lon</u>$^+$ (a - d) and <u>lon</u>$^-$ (e - h) cells in F- medium. Magnification a - f, approx. 1150X; g - h, approx. 460X.

a. 0 min, <u>lon</u>$^+$ cells - normal sized cells

b. 180 min, <u>lon</u>$^+$ cells - cells enlarged but still dividing

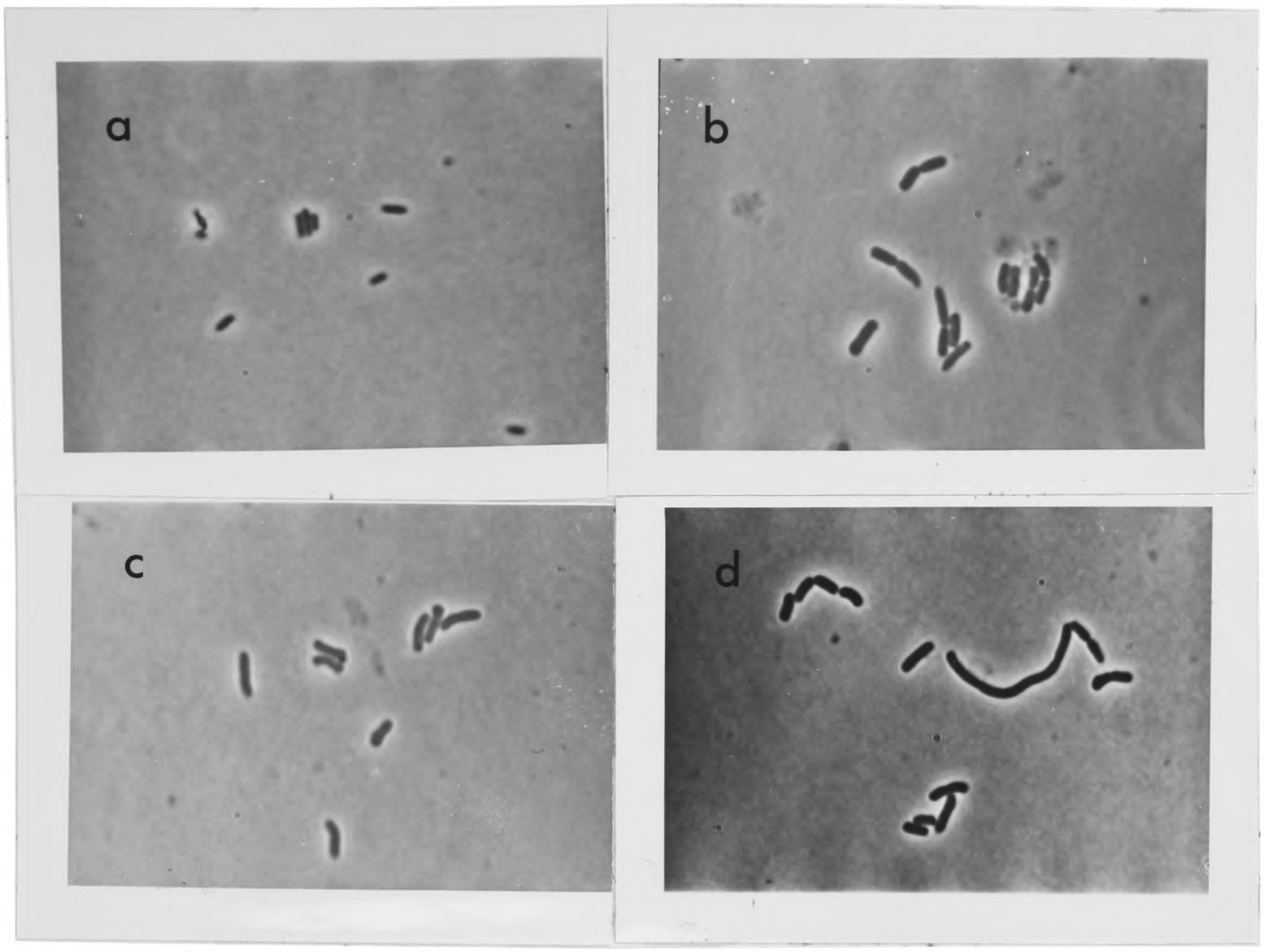
c. 240 min & d, 300 min, <u>lon</u>$^+$ cells - no further increase in cell size. Division continues.

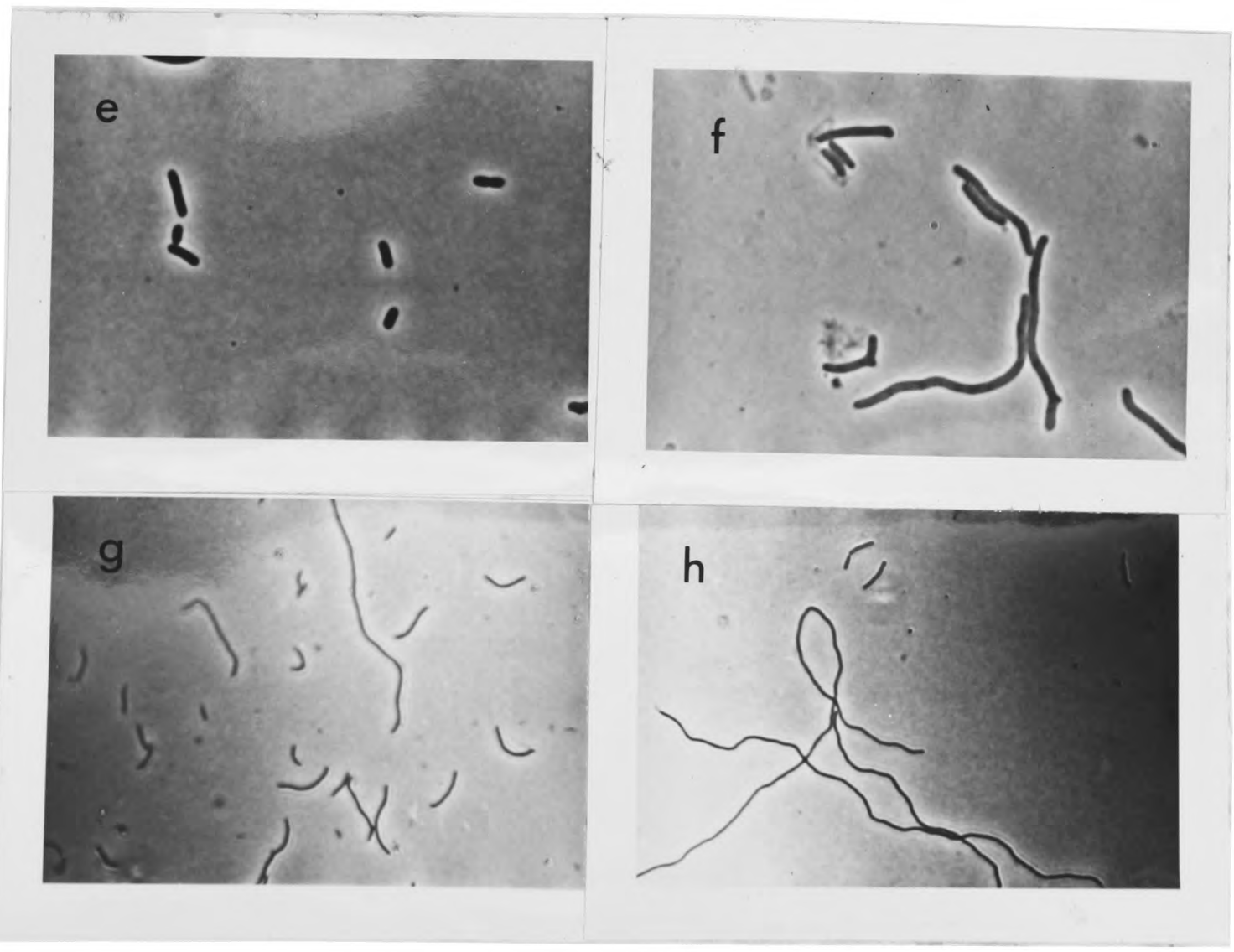
e. 0 min, <u>lon</u>$^-$ cells - normal sized cells, slightly larger than <u>lon</u>$^+$ cells.

f. 180 min, <u>lon</u>$^-$ cells - filaments forming and two cells also show branches beginning to form.

g. 240 min & h, 300 min, <u>lon</u>$^-$ cells - although some cells do retain the ability to divide, many have lost it and filaments continue to increase in size.

Fig. XVI





density during growth in F-medium was followed, the growth rate was slower in F-medium (50 min (lon$^+$), 60-76 min (lon$^-$)) than in nutrient broth (20 min (lon$^+$), 22 min (lon$^-$)). Not all lon$^-$ cells in the culture filamented and there was a slow increase in cell number in F-medium (doubling time 126 min), presumably due to division of the normal cells.

Pantoyl lactone has been shown to reverse filamentation in lon by slowing growth. If pantoyl lactone (0.08M) was added to a culture of F-medium - induced lon filaments, after a brief period of growth, lysis occurred.

Weinbaum (1970) found that in F-medium, filaments were often branched, whereas only casein hydrolysate (CAA) needed to be added to the broth to obtain unbranched filaments (I observed few branched filaments in F-medium). The effect of casamino acids on lon$^-$ cells was examined. Table III shows the composition of the various media used and the growth characteristics of lon$^-$ cells in them.

F-medium has a greater effect on lon$^-$ cells than on lon$^+$ cells but, as evidenced by the very long filaments, growth continues initially although the cells are unable to divide. That this is a property of the casein hydrolysate may be seen by examining the effect of the other media on lon$^-$ cells. F3 medium, which is minimal medium enriched with 5% casein hydrolysate has the same effect on the cells as F-medium. Even minimal casamino acid

TABLE III

Constituents of Media

Organism	Medium	Growth	Nutrient Broth	Minimal Medium	%CAA	NaCl + lysine	trp + uracil	Microscopic Appearance
AB1157	NB	+++	+	-	-	-	-	normal cells
	F	+++	+	-	5	+	-	normal cells in clumps, some spheroplasts
AB1899NM	NB	+++	+	-	-	-	-	normal cells, a few filaments
	MCA	++	-	+	0.2	-	+	normal cells, many filaments
	F	-	+	-	5	+	-	very long filaments
	F2*	++	+	-	5	-	-	mainly normal cells but many filaments
	F3**	+	-	+	5	-	-	very long filaments
	F4***	-	-	+	5	-	+	very long filaments
	F5****	+++	+	-	-	+	-	mainly small cells, but many filaments

* nutrient broth + 5% casamino acids

** minimal medium + 5% casamino acids

*** minimal casamino acids medium with 5% casamino acids instead of 0.2%

**** F-medium without casamino acids

NB = nutrient broth; MCA = minimal casamino acids medium.

medium (MCA) with only 0.2% CAA increases the number of filaments seen in an overnight culture, and if the casamino acid concentration is increased to 5% (F4 medium), the effect is the same as with F-medium. Adding 5% CAA to nutrient broth inhibits division, although not to the same extent as when it was added to minimal medium. Possibly the broth has some protective property for the cells. If casamino acids are omitted from F-medium, some filamentation still occurs but the effect is very reduced.

These results all suggest a surface change induced by these media in both lon$^+$ and lon$^-$ cells. The effect of this change on lon$^+$ cells is small, but in lon$^-$ cells, it prevents division.

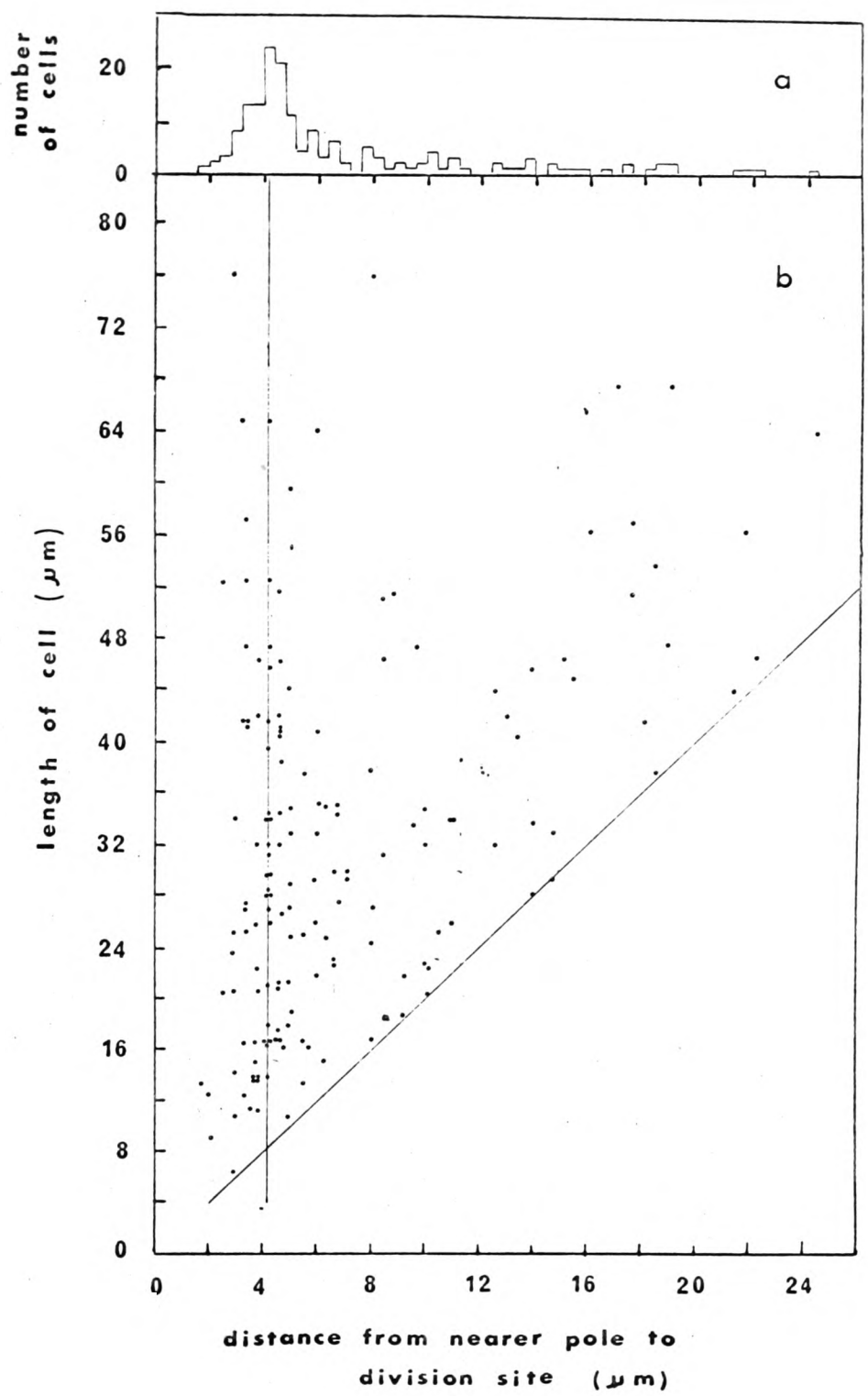
Division Sites after Thymine Starvation: One possible explanation for the delay in division of lon$^-$ cells after thymine starvation is that the nascent division site is destroyed by inhibition of DNA synthesis and division must wait for a new site to be synthesized. Examination of the model of Donachie and Begg (36) indicates that the oldest division site in a cell is the central site. This would mean that after thymine starvation, none of the cells would divide centrally. A lon$^-$ culture was starved of thymine for 80 min, thymine readded, and the cells transferred to minimal soft agar on a slide. The slide was maintained at 37° and growth of the cells was observed through a microscope. Photographs were taken of the dividing cells and the length of the cell and the distance of the division site from the

closest end of the cell measured. Very few cells divided centrally (Fig. XVII) nor did the division sites fit any other of Donachie's predicted sites (25 , $12\frac{1}{2}$ or $37\frac{1}{2}$% of the length of the cell, as are observed in filaments of *lon*$^+$ strains). There is a very marked tendency, however, for divisions to occur 3-5μ from one end. This is approximately 2-3 'unit-cells' using the length of 1.7μ as the 'unit cell' as found by Donachie and Begg for *E.coli* 15T$^-$ & B/r (36). The figure is likely to be similar for these cells also since the smallest *lon*$^+$ (Fig XVIII) or *lon*$^-$ cell found was 1.7μ long. This means that not only does the cell not use the division site formed during thymine starvation but it always tries to divide off a cell 2-3 'unit cells' in length. This way of dividing requires that the cell have some method of measuring this length from one of its poles, and of choosing this site in preference to sites which would be predicted from the model of Donachie and Begg (36) even though the two sites may be close together.

Relationship Between Lon and a Change in the Cell Envelope:

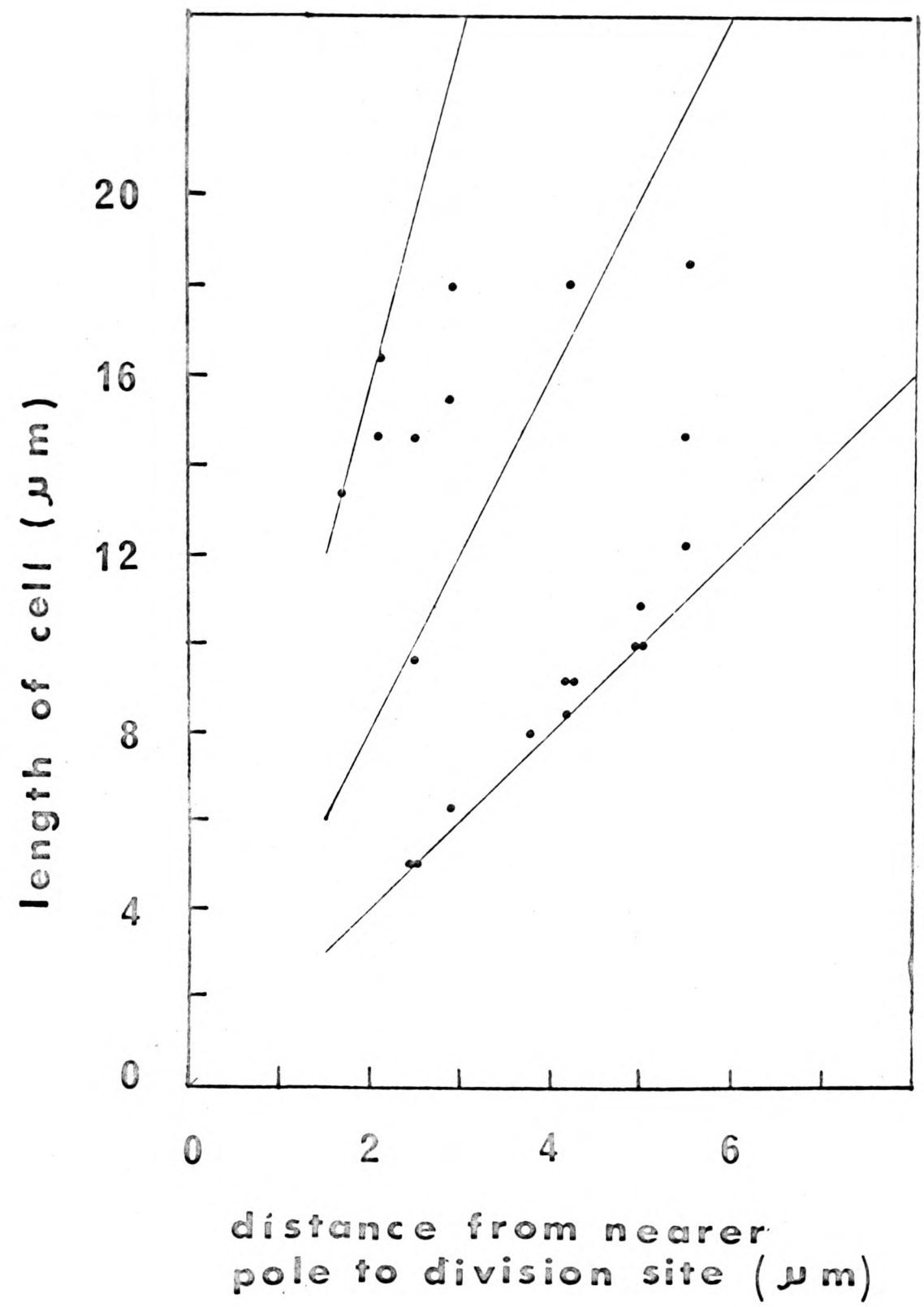
The properties of the *lon*-mutation suggested that it could be a membrane defect. The bacterial nucleus appears to be connected to the cell membrane (21, 55, 114, 130, 132) and it is possible that the DNA attachment site is also the site of cell division. Inhibition of DNA synthesis might cause a change in the membrane at this position with the resultant delay or inhibition of cell division. A relationship between inhibition of DNA synthesis and membrane changes has already been reported (78, 79). Inouye and

Fig. XVII



Positions of division sites in lon⁻ cells after thymine starvation. TG894, lon⁻ cells were starved of thymine for 60 min. Thymine was readded and the cells photographed when they divided. a. Distribution of distances of division sites from nearest pole. b. Plot of distance of division site from nearer pole with respect to total length of cell. The diagonal line represents the positions of median division sites. The vertical line indicates the preferred position for division site.

Fig. XVIII



Positions of division sites in <u>lon</u>$^{+}$ cells after thymine starvation. Experimental procedure as Fig. XVII. The diagonal lines represent the position of division sites located at 50, 25 and 12½% of the cell length.

Guthrie (78) used a temperature sensitive mutant of E.coli which was able to divide but unable to replicate its DNA at 41°. At the elevated temperature, a change was found in the membrane protein fraction, although both mutant and parent showed the same pattern at 28°. Subsequently, Inouye and Pardee (79) showed that changes occurred in the membrane proteins when DNA synthesis and cell division were inhibited in various ways, and it was this which they had initially observed. I decided, therefore, to see whether untreated lon$^-$ cells possessed a change in the membrane protein as compared to lon$^+$ cells.

Mutants of E.coli have been described which adsorb colicin but are resistant to its action (105, 108). Their resistance appears to be due to a change in a surface component (105). These mutants were found to be sensitive to sodium deoxycholate, although the action of this chemical was not fully understood. AB1899NM (lon$^-$) and AB1157 (lon$^+$) were tested for their sensitivity to deoxycholate, but both were found to be equally resistant.

Mapping only about 0.3 min away from lon is the gene acrA which controls sensitivity to acriflavine. This gene is believed to determine the synthesis of a membrane component (106). Wild-type strains of E.coli K12 are resistant to acriflavine but strains mutant at this gene (acrA$^-$) are sensitive to it. Again, the lon$^+$ and lon$^-$ strains were tested for sensitivity and the results are seen in Table IV. The lon$^-$ cells show a greater sensitivity to acriflavine than wild-type cells. This is suggestive

TABLE IV

Strain	Viable Count of Untreated Cells	Viable Count of Treated Cells	Per Cent Survivors
AB1157 lon$^+$	5.3×10^8	4.0×10^8	75
	8.2×10^8	6.3×10^8	77
AB1899NM lon$^-$	5.9×10^8	2.6×10^8	44
	4.7×10^8	0.9×10^8	19

of a defect in the membrane of the mutant, but the increased sensitivity is slight and few conclusions can be drawn. There is no indication that lon$^-$ cells are also acrA$^-$. Rather, these results would suggest that the acrA - phenotype may be partially mimiced by the lon - phenotype. That is, the mutant lon-gene may cause a slight increase in the permeability of membrane to acriflavine.

There have been several studies by acrylamide gel electrophoresis of the protein composition of the membrane of Gram-negative organisms (78, 79, 134, 136, 138). If the lon-mutation does cause a change in a membrane protein, it is possible that this would be revealed by running a labelled membrane preparation on acrylamide gel.

Electrophoresis using acrylamide gel is largely a modification of the older technique of paper electrophoresis. The paper as a stabilizing medium for the proteins has been replaced by a polyacrylamide gel held in a tube. Each end of the tube is immersed in buffer and a current passed through the gels by electrodes placed in each buffer reservoir. The one major difference between paper electrophoresis and acrylamide gel electrophoresis is that whereas using paper, the proteins are separated only on the basis of charge, with the gel, there is an additional sieving effect. The pore size of the gel can be varied by varying the concentration of acrylamide, so that molecules larger than a certain size can be excluded.

The gel is made up of five reagents. The acrylamide monomer and its cross-linking agent (methylenebisacrylamide or ethylene diacrylate), a buffer solution, tetramethylethylenediamine (TEMED), which is the initiator of polymerization, and ammonium persulphate, the catalyst. The preparation of the running gels has been described in Chapter 2. Originally the gel was over-layed with a small volume of gel with larger pore size (spacer gel) and the sample mixed with 0.2ml of this large-pore gel (sample gel) and applied on top of the spacer gel. The purpose was to concentrate the sample in a narrow band at the top of the small pore gel since all proteins would be held up at the inter-face of the two gels. However, as pointed out by Raymond (119), the "thinness of the sample zone depends on the ratio of migration velocity in the sample gel to migration velocity in the supporting (running) gel. The lower the concentration of the sample gel, the higher this migration ratio will be. Therefore the maximum degree of thinness in the starting zone will be achieved when the sample gel is omitted entirely". This is achieved by adding the sample in a sucrose solution which, due to its high density, settles on the top of the running gel. The only purpose achieved by the spacer gel which is not satisfied by sucrose is that the proteins begin stacking into discs in the spacer gel already before entering the running gel.

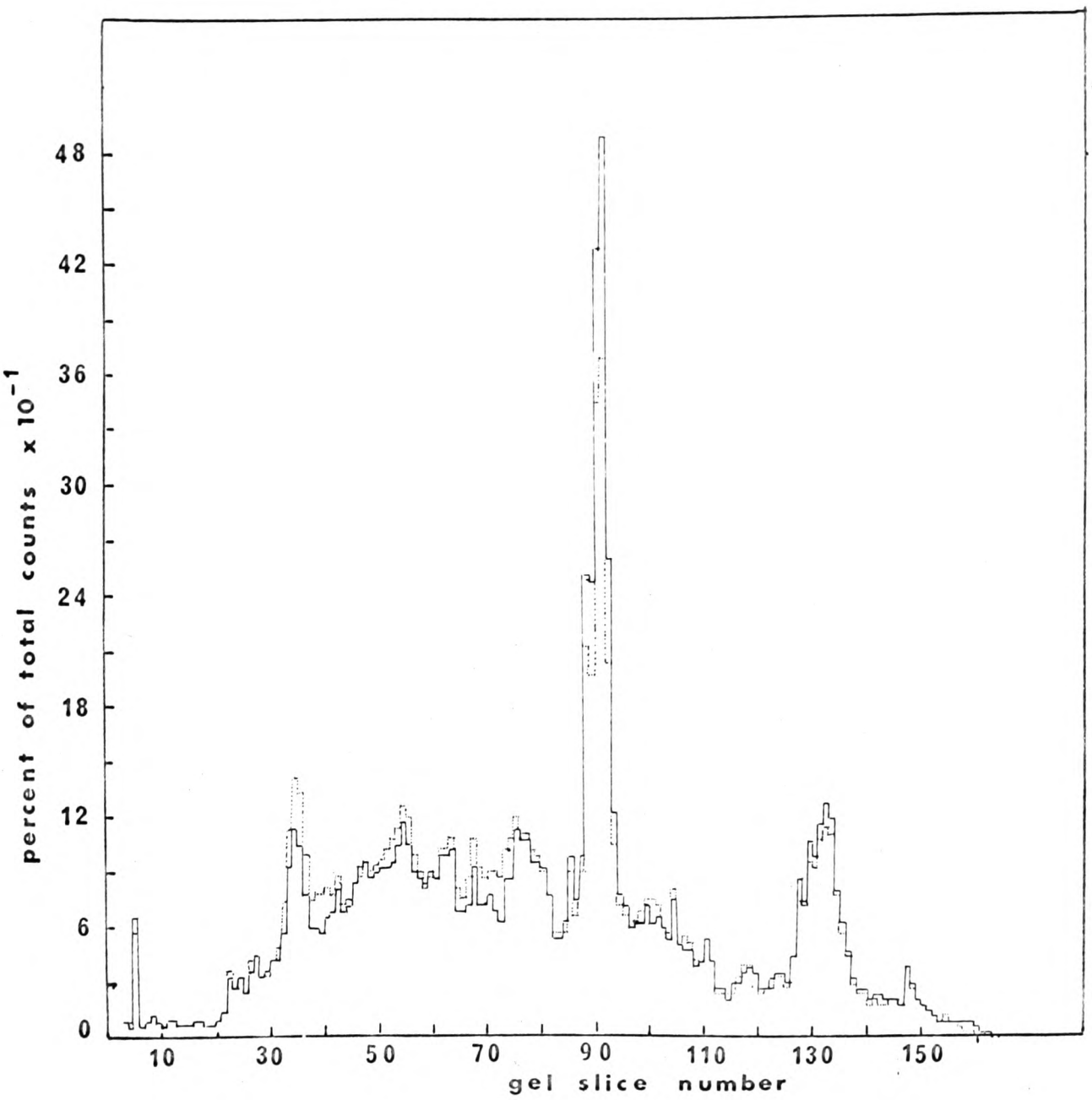
$\underline{Lon}^+$ cells were grown in the presence of ^{14}C - leucine and $\underline{lon}^-$ cells in ^{3}H - leucine. These cultures were mixed and

the envelope fraction separated and dissolved. The solubilized envelope was run on an acrylamide gel which was then sliced and counted for ^{3}H - ^{14}C discrimination. The results are seen in Fig XIX. A similar experiment was also performed with the labels reversed and the results of this are seen in Fig. XX. In both figures, the percentage of the total counts in the major peak centering around slice number 92 is different for the two strains. In Fig. XIX, this peak shows a decrease from 20.5% of the total counts (slices 88-96) in the wild-type to 16.5% of the total counts in the mutant. In Fig. XX, the corresponding decrease (slices 89-97) show a decrease in the percent of total counts in the peak from 16.5% (lon^{+}) to 14.5% (lon^{-}). This is the only significant difference between the two strains. All other differences in peak volume are likely to be due to chance. This peak appears to correspond to the Z peak of Inouye and Pardee (79). They did not suggest a function for their Z peak, but since it appears to be decreased by thymidine starvation or UV-irradiation, it is likely to be connected with DNA replication or cell division. Schnaitman used the double labelling technique to compare the gel electrophoresis pattern of proteins from both cell wall and cytoplasmic membrane (137). Comparison of his results and my own suggest that my major peak corresponds with his peak number 16. This peak, he suggests, is the major protein of cell wall, and is the structural protein of the membranous component of the wall.

The gels show 16 'peaks', but it is probable that these could be further resolved. It is likely that the peaks near the top of the gel are membrane fractions not sufficiently dissociated

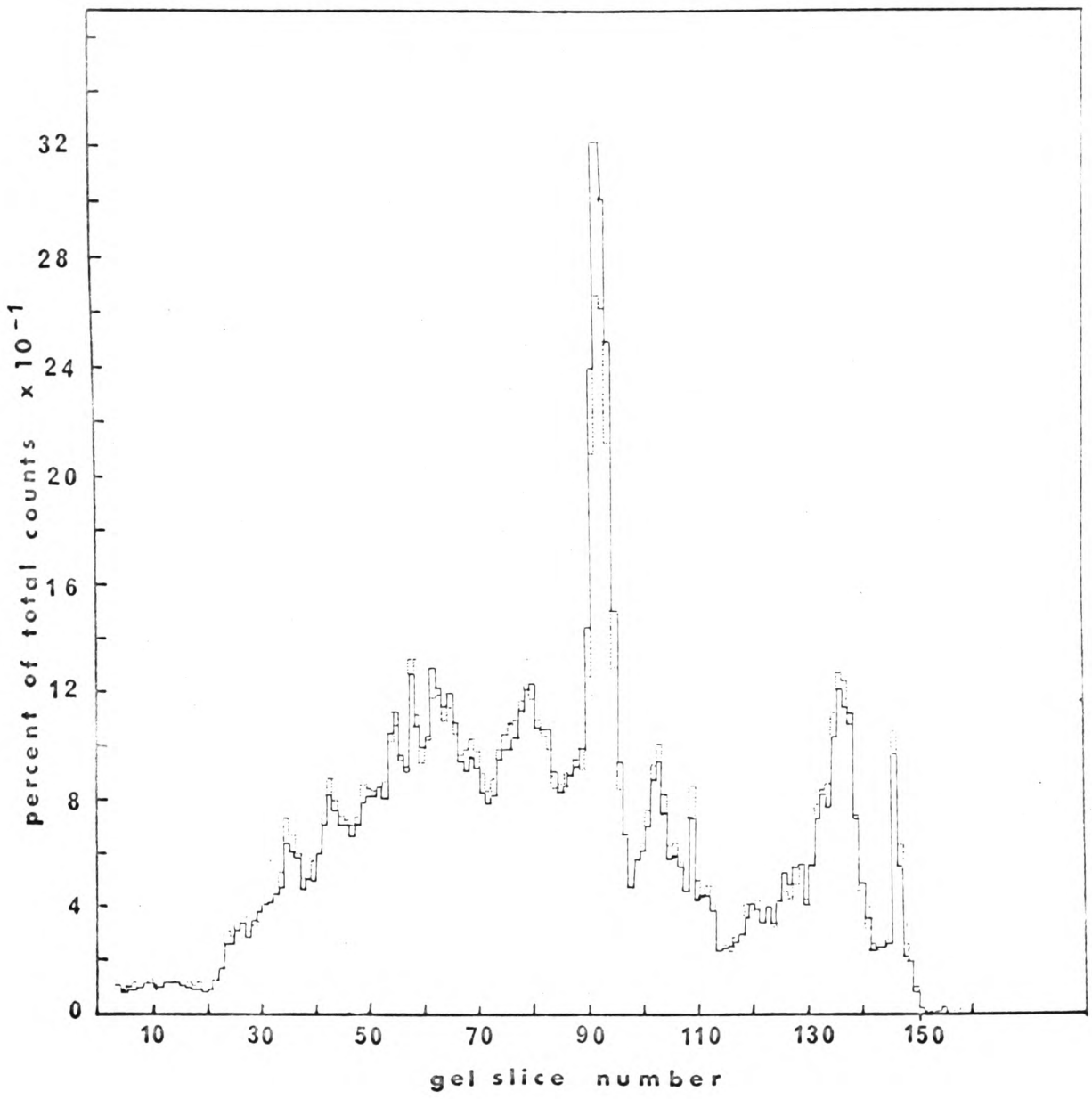
to pass through the gel. Due to the density of the gel, much material was trapped in the top 2-3 slices (top 2-3 mm of gel) and these have been ignored for the purpose of calculations and plotting the results.

Fig. XIX



Acrylamide gel electrophoresis of envelope proteins. The solubilized envelope proteins were prepared and run as described in Chapter 2. ^{14}C-labelled AB1157, lon$^+$ - solid line; ^3H-labelled AB1899NM, lon$^-$ - dotted line. The counts of each gel slice are plotted as a percentage of the total counts in the gel. The top 3 slices containing material too large to migrate through the gel has not been counted for the purpose of these calculations.

Fig. XX



As Fig. XIX. ^{3}H-labelled AB1157, lon^{+} – solid line; ^{14}C-labelled AB1899NM, lon^{-} – dotted line.

CHAPTER 4 : CONCLUSIONS

One way to obtain more information about the normal cell is to study the abnormal cell. Lon-mutants of E.coli possess a very sensitive cell division mechanism which is disrupted by inhibition of DNA synthesis. In this study of the lon-mutation, I have tried to discover more both about the mechanism of action of the mutation itself and about cell division in E.coli in general.

The lon-mutant of E.coli was originally identified as a UV-sensitive mutant (33, 32). It is now apparent that the organism is sensitive to the DNA inhibition caused by UV-irradiation, and the effect can be mimiced by many other treatments. I have now shown that thymine starvation can induce filamentation in lon cells. Since the sole action of removal of thymine is to prevent DNA synthesis, inhibition of chromosomal replication is unequivocally linked to filamentation in lon$^-$ cells. It is true that the breakdown of the division process may be due to a consequence of inhibition of DNA synthesis. For example, thymine starvation results in a reduction in the thymidine (dTTP) pool of the cell. To maintain its deoxynucleoside triphosphate pool constant, there is a consequent increase in the dATP pool (107). It is possible that it is the increase in the dATP pool which causes inhibition of division in these mutants. This is unlikely, however, since it does not explain the pleiotropic effect of the lon-gene in causing mucoidity, the induction of filaments by

nutritional means, or the selectivity as to the division site used after thymine starvation.

First I will look at various possible explanations for the long delay in cell division in *lon*$^-$ cells, and show how my results do not support them. I will then describe a model for the *lon* mutation and examine it in the light of my results.

The first theory which I examined was that the *lon*$^-$ cells failed to recover their DNA/mass ratio after inhibition of DNA synthesis. This could occur if the mutant cells, unlike the wild type, did not reinitiate after thymine starvation or UV-irradiation (14, 15, 116). The cell mass increases normally during DNA inhibition, and so if, when DNA replication restarts, it does so at the old rate (i.e. the same rate of increase as cell mass), it can never catch up with the cell mass. This was not found to be the case. The *lon*$^+$ and *lon*$^-$ cultures showed similar periods of rapid DNA synthesis after both thymine starvation (Fig.V) and UV-irradiation (Fig VI) so that the cells possessed their normal complement of DNA within 40-60 min. A different situation is seen if UV-irradiated cells are 'stepped-up' after irradiation (Fig VII). Once again, when DNA synthesis restarts, it does so at a rapid rate, however, due to the step-up, the cell mass is also increasing at a faster rate. Since the cells do not appear to regain their normal DNA complement, it is likely that they have failed to initiate sufficient rounds of replication. In this case, the cells would be expected to filament since they would never

attain a normal DNA/mass ratio, and so never receive the stimulus to divide. An experiment to examine cell division after UV irradiation followed by a step-up is being planned.

After thymine starvation for one mass doubling time, both lon$^+$ and lon$^-$ cells regain their normal DNA content in about 40 min. This is also the time that lon$^+$ cells start dividing after thymine starvation (Fig V & VIII). It appears, therefore, that that is the main criterion for these cells to divide and restoration of the DNA/mass ratio is the final step before division can occur. This is not the case for lon$^-$ cells which are delayed a further 75 min before they divide (Fig VIII). The question of what occurs in these 75 min is discussed later in this section in terms of the model which I am proposing. The same picture is seen after UV irradiation of lon$^-$ cells (Fig. IX).

The 45 min delay in division of lon$^+$ cells after thymine starvation is a maximum reached after growth for 80 min in the absence of thymine. Since the delay in division is shorter than the starvation period, many of the stages of cell division will have been completed during the starvation period or can be completed within the 45 min. This delay may be explained by saying that the chromosome reinitiates at both origins in the cell and that cell division must then wait for completion of these rounds of replication, a process which will take about 45 min. Similarly, the 120 min delay in division of lon$^-$ cells is a maximum. Since these cells also regain their DNA content in 45 min, the explanation is not that it takes 120 min to restore the DNA content of the mutant cells.

Previously it was thought that lon$^-$ cells lost the ability to divide after inhibition of chromosome replication. These results indicate (and have been confirmed (166)) that rather than being inhibited, cell division in these cells is delayed. With increased length of DNA inhibition, there is reduced survival of cells. This could be explained by the cells possessing a critical length and cells which exceed this length cannot usually divide. Since an untreated culture of lon$^-$ cells contains filaments and elongated cells, survival of a lon$^-$ culture would begin to decrease before that of a lon$^+$ culture which consists completely of normal sized cells. With long periods of thymine starvation followed by the 120 min delay before division occurs, many lon$^-$ cells would be killed. To test this hypothesis, lon$^-$ cells were made to filament in low concentrations of penicillin before they were grown in the absence of thymine. With short periods of penicillin treatment, there was no difference in the delay before cell division after thymine starvation as compared to cells starved of thymine only (Table II). Long periods of penicillin treatment, however, delay division considerably and may inhibit it completely. This would appear to support the hypothesis since only cell division has been affected in these cells causing them to filament instead of dividing after each doubling of cell mass. It is possible that growing for 120 min in penicillin caused deficiencies in the cell wall which made the cells sensitive to removal of thymine. However, Stárka and Moravová (151) found that E.coli B could be treated for 150 min

with low concentrations of penicillin with no ill effects.

It has been suggested that filamentation is due to accumulation of an inhibitor of cell division (36, 61, 141, 176) or by deficiency of an activator of division (61, 141). If the latter were the case, amino acid starvation would lengthen the delay period before division occurred (after readdition of amino acids) by the length of time necessary for synthesis of this activator (which appears to from my results be 120 min). This was not found to be so (Fig XII). As mentioned below, my results do support the idea of an 'inhibitor' of cell division.

These results may be explained by saying that lon$^-$ cells normally possess a longer C time than lon$^+$ cells and that amino acid starvation slows the rate of travel of the replication point 2-2.5 fold. However, there is still another difference between the lon$^+$ and lon$^-$ cells. Whereas in lon$^+$ cells, this relationship holds even to 0 min amino acid starvation, with lon$^-$ cells, there is a 120 min delay during which DNA replication continues at the slow rate but which is not decreased by this DNA synthesis (which would be completed before 120 min). This delay I explain as being the time required for decay of an inhibitor of cell division. However it may be seen that division depends not only on decay of this inhibitor but also requires termination of the chromosome. If division depended solely on decay of the inhibitor, there would be no further delay in division after 120 min of amino acid starvation.

Thymine starvation inhibits DNA replication, and since termination of a round of replication is a necessary stimulus for cell division, this also ceases. It was possible that in *lon*$^-$ cells, once the cell division process was stopped, it was restarted with difficulty. Inhibition of DNA replication would then be exerting its effect on *lon*$^-$ cells by inhibiting cell division. This possibility could be tested by inhibiting division without affecting chromosome replication. Low concentrations of penicillin possess the property of preventing cell division without affecting growth or nuclear replication. *Lon*$^+$ and *lon*$^-$ cells behave similarly (Fig XIII, XIV A) with division occurring 35-50 min after the addition of penicillinase. Moreover, if the culture is thymine starved during penicillin treatment (Fig XIV B), the cells divide at the time which would be expected for thymine starvation alone. Therefore, penicillin treatment neither prevents expression of the *lon*-phenotype nor enhances the delay in cell division found in these cells after thymine starvation.

I would like to propose a different theory to explain the effect of the *lon* gene, and I will then see to what extent my results support it. It has been assumed up to now that termination of a round of replication acted as an initiator of cell division. W. Donachie (pers. commun.) has suggested that the control mechanism may be a purely physical one. He proposed that when DNA replication terminates, the chromosome is released from the cell

membrane. If chromosome replication is inhibited, it does not leave the membrane and cell division is physically blocked. When thymine is readded to thymine-starved lon$^+$ cells, chromosome replication continues, and on termination, the chromosome detaches from the membrane and cross-wall formation occurs. Possibly in lon$^-$ cells, septum formation ceases during inhibition of DNA synthesis and cannot be restarted when DNA synthesis continues. The division site affected is the central division site of the cell which consequently is not used (see Fig XVII). From this figure, it may be seen that for cells 10-30μ (6-18 'unit cells'), division is almost completely restricted to a site 2-3 'unit cells' from one end. Larger cells show a definite preference for this position, but divisions do occur elsewhere in the cell. Division at these other sites is prevented by formation of an inhibitor of division during thymine starvation. This inhibitor takes 120 min to decay.

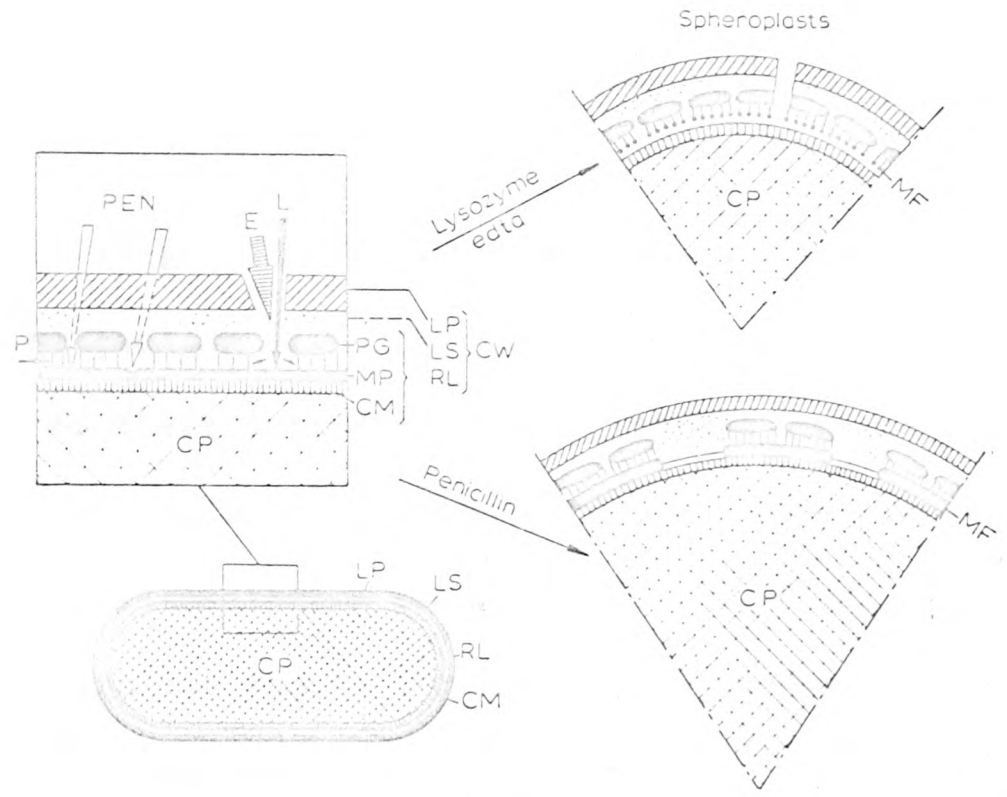
In summary, thymine starvation prevents separation of the chromosome from the membrane, resulting in a change in the cell envelope at the site of DNA attachment to the membrane, which in lon$^+$ cells is also the future division site. The change inhibits septum formation at this site in lon$^-$ cells. Division at other sites is prevented until after decay of an inhibitor. I will now see how this theory is supported by my data.

The lon gene not only causes sensitivity to inhibition of DNA synthesis but also causes the cell to possess a large capsule

so that colonies on minimal agar appear mucoid. The organism used in this work was non-mucoid, but it possesses a mutation in a structural gene for polysaccharide (97). This leakage of excess capsular material suggests a defect in the cell envelope. The cells were also found by acrylamide gel electrophoresis to be relatively deficient in cell wall material, again suggesting a defect in their envelopes. A brief description of the structure of the bacterial envelope (see Fig XXI) would be useful at this point. "The majority of the thin sections of Gram-negative bacteria show multilayered parallel 'membranes' in the envelope structure. The outermost structure has usually been referred to as the wall and the inner one as the plasma or cytoplasmic membrane" (133). The cell wall is composed of an outer layer of spherical macromolecular components (lipopolysaccharide-protein) and an inner rigid continuous layer of glycosaminopeptide or mucopeptide (murein) (133, 137). There are several other layers outside the cell wall, but I will not be concerned with them.

The envelopes of lon$^+$ and lon$^-$ cells were solubilized and run on acrylamide gels and the profiles compared. The mutant appears to possess a decrease in the major protein component of (possibly) the external lipopolysaccharide-protein layer. The observation that the protein is reduced rather than absent is understandable in the light of the fact that the mutant grows normally unless treated in some manner. That the change in lon$^-$ cells is due to a change in the outer layer of the cell wall explains several

Fig. XXI (from Salton (133))



A diagrammatic representation of a Gram-negative bacterium with a complex, triple-layered cel wall. Separation of the layers can be achieved by solvent extraction and by treatment with proteolytic enzyme (P). Both lysozyme (L), aided by EDTA (E), and penicillin induce depolymerization of the rigid mucopolymer (MP), although probably to a different degree and in a different way. Only small mucopolymer fragments (MF), which are covalently linked to other cell wall components, remain in the wall after lysozyme treatment. Penicillin spheroplasts and the related L-forms may retain larger mucopolymer fragments or a modified, non-rigid mucopolymer in their stretched cell walls which surround protoplasts of greatly increased dimensions. Abbreviations used: LP = lipoprotein layer, LS = lipopolysaccharide layer, RL = rigid layer; PG = protein granula, MP = mucopolymer, CM = cytoplasmic membrane, CP = cytoplasm, MF = mucopolymer fragments, PEN = penicillin, L = lysozyme, E = ethylenediamine-tetraacetic acid, P = proteolytic enzyme. (Schematic model devised by MARTIN, 1963).

of the observations. The outer layer of the wall is likely to play a role in the permeability of the cell to its surrounding medium. Since growth does not appear to be affected by the mutation and growth rates are similar for wild-type and mutant, there does not appear to be any major change in permeability. Nevertheless, lon$^-$ cells have been shown to be slightly more sensitive to acriflavine than wild-type cells.

Many of the results may be explained by a change in the cell metabolism being due to a change at the cell surface. The first of these is the effect of pantoyl lactone and F-medium on the cell. Pantoyl lactone has been found to reverse and prevent filamentation of lon$^-$ cells, however, no report has appeared explaining the reason for this. My results indicate that its action on the cell is to slow growth. Media with a high concentration of casein hydrolysate (F-medium) cause filamentation in lon$^-$ cells and clumping and spheroplast formation in lon$^+$ cells. Growth is also retarded in this medium. A common way of obtaining spheroplasts is to treat cells with a high concentration of penicillin. Since the action of the penicillin is to prevent the formation of fully cross-linked cell walls, it is not unlikely that the F-medium also induces discontinuities in the cell wall. With the lon$^-$ cells, the effect is more drastic and no division at all can occur. Since this medium acts differently on the two types of cells, it would suggest that the envelope of the mutant is slightly different to that of the wild-type. If pantoyl lactone is added to these lon-filaments, lysis occurs. This is further

indication that both pantoyl lactone and the F-medium act on the bacterial envelope. One question raised by these results for which there is no answer at this time is why pantoyl lactone, which inhibits UV or crystal violet-induced lysis of B. subtilis (160) and enhances cell septation should cause lysis of these filaments.

Observation of the growth of lon$^-$ cells recovering from thymine starvation revealed anomalies in the sites of division. Few lon$^+$ cells were measured but even the few points indicate a preference for the central site for division. (10/20 of the points fall on the 50% line). Other work with 15T$^-$ and B/r (36) indicates that long cells possess sites 50, 37½, 25 and 12½% from the nearest pole (longer cells will show more sites). The lon$^-$ cells have lost the ability to use these sites (at least initially) and instead always use a site 2-3 'unit cell' lengths from one end. This again points to an effect on the cell membrane or wall.

Steed and Murray (152) reported that E.coli forms a membranous septum before cross-wall growth occurs. Deering and Setlow claimed (32) that if UV induced filaments of lon$^-$ cells were stained for cytoplasmic cell boundaries, dark staining bands were seen, one cell length apart along the filament. Division occasionally occurred at these bands. This suggested that the lon-mutation may result in the inability to form a cross-wall although septum formation occurs normally. The filaments would

be, in effect, chains of compartments separated by cell septa but division would be unable to be completed. Thymine-induced filaments were examined by means of the electron microscope to see if these septa are present.

It was thought likely that the reason why these septa were seen in E.coli by Steed & Murray only under certain conditions is that the incomplete septum is labile. Under the conditions they used, the septum was stabilized and so became viable. It was thought that the complete septa, which would be present in lon$^-$ cells if they were unaffected by inhibition of DNA synthesis would not be labile and so should be visible under normal fixation methods. No septa were seen in the cells 80 min after readdition of thymine to the medium. For technical reasons, it was not possible to fix the cells in the manner Steed & Murray did, and the assumption has been made that a complete membrane is stable to the normal fixation methods used for the electron microscope. Since no septa were seen, this would mean that they were incomplete and were lost during fixation, and since these sites subsequently became inactive in division, it is likely that no new septum formed there when thymine was readded.

To restate the hypothesis, the lon$^-$ gene causes a defect in the cell envelope. Under normal growth conditions, the only effect on the cell is to slow down the rate of travel of the replication point so that the C time is about 87 min. In both lon$^+$

and lon⁻ cells, temporary inhibition of DNA synthesis prevents separation of the chromosome from the membrane. This separation is a prerequisite for division site formation. In lon⁺ cells, this is a temporary effect and if DNA replication is allowed to terminate, division occurs normally. In lon⁻ cells, the attachment of the DNA to the membrane during inhibition of DNA synthesis causes a change in the envelope. This change prevents septum formation and this central site can not be used as a division site. The change in the envelope due to inhibition of DNA synthesis also produces inhibition of cell division at other sites in the cell, even when DNA replication restarts. The maximum amount of 'inhibitor' is accumulated after 60 min thymine starvation, and this amount takes 120 min to decay once DNA synthesis restarts. Thymine starvation for periods less than 60 min produces less 'inhibitor' and the time for its decay is less than 120 min. The amount of 'inhibitor' produced is directly proportional to the time of DNA inhibition over the period 0 - 25 min DNA inhibition (0 - 90 min delay in division). When the inhibitor has decayed, division occurs preferentially at a site 2 - 3 'unit cells' from one pole. Possibly, with time, the central site can also recover. A medium which appears to affect the cell surface can induce a similar change in the envelope, so inhibiting division of lon⁻ cells. Division is also dependent on termination of chromosome replication. If the time for termination is greater than the time for decay of the 'inhibitor', then division is delayed until chromosome rounds are completed.

The final point I would like to examine is how much more is known now about the mechanism of division? This process is now seen to have several stages, although our knowledge is not great enough at this time to give a precise sequence to these stages, and the order I give is likely to be subject to alteration. I will examine several treatments which affect cell division to see what is known about each. I will then fit them into a sequence which I believe may be the order of their effects on cell division.

Smith and Pardee (147) described a heat-labile protein required for division which accumulates during most of the cell cycle. It is destroyed at 45° which delays division up to a maximum of 25-30 min. The authors suggested that this protein is the septum itself. Since it begins to accumulate shortly after cell division, it may be considered the first step of the following cell division. Septum formation is not linked to DNA replication since the two are discoordinated by the heat shock. Steed and Murray (152) observed a septum preceding visible invagination of the cell wall using modified electron microscope techniques. Presumably this stage can also be inhibited by any inhibitor of protein synthesis.

Penicillin in very low concentrations inhibits division but not growth. Since its action, as far as is known, is solely on the cell wall, it may be that it causes discontinuities in the wall which prevent the changes described below from occurring.

When penicillin is added to a culture, the amount of residual division depends on the concentration of the drug, so that it is likely to exert its effect on cells of all ages rather than at a specific time. Similarly, when penicillinase is added, there is a 45 min delay, presumably for repair of the lesions, and cell number then increases exponentially rather than as a burst, indicating that division has not been synchronized in these cells, (i.e. the cells have not all been blocked at one point in the cell cycle.) Therefore, there does nit appear to be any one penicillin sensitive stage in cell division, but rather it acts throughout the cell cycle.

The role that termination may play in division has been discussed earlier. Its timing with respect to cell division is easier to fix and it occurs about 20 min before division (28, 65). The postulated separation of the chromosomes from the cell membrane would be the initiator for changes to occur in the envelope in preparation for cross-wall formation.

Inouye and Pardee (181) found that the putrescine/spermidine ratio had a controlling effect on cell division. Putrescine appears to be necessary for division whilst spermidine is antagonistic, although their specific activities are not known. The polyamine ratio appears to exert its effect about 10 min before division occurs, since it takes 50 min for the ratio to return to normal in cells unable to synthesize polyamines during arginine starvation, and since division occurs 60 min after the

readdition of arginine. Inouye and Pardee suggested that small changes in the ratio may be sufficient to have a significance, and proposed three possible roles for the polyamines.

Cell division now enters the final stages of cross-wall formation and cell separation. Cell separation appears to occur almost simultaneously with cross-wall formation and is the final stage in the division process. The division stages as I have proposed them are:

1. septum formation
2. penicillin-sensitive changes in the cell wall
3. termination of DNA replication - chromosome separates from membrane
4. change in the polyamine ratio removed inhibition from division
5. cross-wall formation
6. cell separation

The first two are processes which are continuous throughout the cell cycle. Septum formation cannot be completed until termination of DNA replication, and similarly penicillin is likely to be effective during much of the cell cycle.

Two models have been presented, one to explain the lon mutation and the other a general pattern for cell division. Both need a lot of further work to be done to see to what extent they are correct.

Electron micrographs of lon filaments growing in the presence of thymine (DNA content restored) show no septa. This suggests that the lon⁻ mutation may prevent formation of the septum after thymine starvation. It is possible that even the completed membrane is unstable to fixation at room temperature and is not being seen for this reason. If this were the case, then the defect caused by the lon gene would be at a later stage in the division cycle, e.g. cross-wall formation.

The other point on which the mode of action of the lon gene, as I have stated it, rests, is the separation of the DNA from the membrane after termination and the attachment of the DNA to the membrane during thymine starvation. This might be examined in two ways. One would be to look for association between DNA and the cell membrane in electron micrographs. Another, and possibly preferable way, is to use biochemical methods to look at this association. The DNA can either be isolated attached to membrane (146, 156) or indirect evidence of the attachment can be sought (50, 185).

The idea of DNA termination deciding the timing of division has received support from these results. If wild-type cells are starved of thymine for one mass doubling time, after a burst of DNA synthesis, which brings the DNA content of the cells back to normal, there is division. If after thymine starvation for one mass doubling time the cells are amino acid starved for greater than 100 min. (if during amino acid starvation the rate of DNA replication decreases), once again division occurs almost immed-

iately after termination.

It may be possible to improve the gel electrophoresis technique for the study of bacterial membrane and wall. The method used in this thesis did not separate the two, making interpretation of any differences obtained difficult and possibly masking small differences. There are improved methods for isolating purified membrane and wall (133, 184) and gel electrophoresis should be applied to these. Variations in the concentration of acrylamide and cross-linking agent will also be tried.

Observation of division in *lon*$^-$ cells recovering from thymine starvation revealed two interesting facts. There was a maximum delay before cell division of 120 min, and when division did occur, it did so at a point two cell lengths from one end. The distribution of cell sizes of *lon*$^-$ cells at the time of division after thymine starvation for 1 mass doubling time (Fig XVII) is about 14-42μ. This size was reached after about 3 mass doubling times, so that the original sizes of the cells were 1.7 - 5.2μ, that is 1-3 'unit cell'lengths. This suggests a tendency for the normal cell to be about 2 'unit cell'lengths long with some dividing and others continuing to grow. Examination of an untreated log phase culture in minimal medium gave a size distribution of 2.1 - 5.9μ. This is understandable in the light of the liklihood that *lon*$^-$ cells possess a long C time (34). This cell length could also explain the tendency for *lon*$^-$ filaments to

divide 2-3 'unit cell's' from one pole. If a cell 2 'unit cell' lengths long doubles its mass and then divides, the new ends will be the sites for further growth which will be in opposite directions (36). If this division does not occur, as in thymine starved lon⁻ filaments, growth may still occur in the same manner with new material being added in the middle of the cell and with conservation of the ends. These ends would be 2 'unit cell' lengths long. Division must wait, however, 120 min for decay of the inhibitor of division.

All experiments were done in minimal medium. The few experiments done in enriched medium gave equivocal results. It would be informative to examine the timing of cell division growing in enriched medium to see whether the length of the delay decreases, indicating that it is tied to growth rate, or whether it remains constant indicating it is independent of growth rate. Long term observation of division of a single cell may also provide further information concerning the growth sites and whether they are temporarily or permanently blocked.

The present methods of inducing filamentation in lon⁻ cells are not ideal. Thymine starvation requires treatment over a period of time whilst other cell functions continue. The same is true for nalidixic acid, which has the added problem that its mode of action is not know. UV irradiation has the disadvantage of being non-specific and may be having multiple effects on the cell apart from affecting DNA synthesis. For this

reason, since the lon gene does appear to have a protein product, it would be advantageous to have a temperature sensitive mutation at this locus. In this case, it would be far easier to test whether or not the lon gene does control septum formation. Also, filaments of the tslon could be induced with limited effect on the cell metabolism.

In these models I have tried to explain as much as possible about the lon gene and cell division. Naturally, whilst they are so theoretical, they will not explain everything. However, since they provide a good explanation for the results of my experiments, I put them forward as a working hypothesis.

The lon$^-$ mutation causes a defect in the cell envelope which has multiple effects on the cell. Acrylamide gel electrophoresis suggests that the envelope of lon$^-$ cells may be different to that of lon$^+$ cells. This has been supported by a recent report (Allen, R.G., N.A.Shafiq & J.R.Walker, (1971), Bact. Proc., p.25) that during treatment with crystal violet, lon$^-$ cells lose the ability to regulate murein synthesis relative to other syntheses. As a result of this envelope defect, the C time of lon$^-$ cells appears to be longer than that of lon$^+$ cells. This is suggested by the long delay in division when thymine starvation is followed by amino acid starvation (see p.53). A more direct measure of the C time must be made by determining the amount of residual DNA synthesis during amino acid starvation. This will give an indication of the number of replication points

present initially, and from this, the C-time can be calculated. There is a further problem of whether the cells were affected by growth being in only 10µg/ml thymine, however, this will affect the absolute number of replication forks and not the relative number in lon$^+$ and lon$^-$ cells.

A second effect of the membrane change is that cell division is inhibited and the decay of this inhibition determines the delay after thymine starvation. Longer periods of starvation result in more inhibitor being produced and a longer time being needed for its decay. A maximum delay of 120 min is reached after 60 min thymine starvation. Examination of Fig X does indeed show that for 0-25 min starvation, doubling of the time of starvation doubles the delay period. This decay is also indicated in Fig XII. Here the lon$^-$ cells show, initially, a delay of 120 min irrespective of when termination occurs.

It has been suggested that thymine starvation prevents separation of the chromosome from the membrane, which is a prerequisite for cell division. I have postulated that whereas in wild-type cells this inhibition is rapidly reversible and cell division occurs at this site when thymine is restored, in lon$^-$ cells reversal of the inhibition takes some time. My results indicate that the central division site, which is the cell's oldest site is not used, at least initially. Experiments will have to be done to test whether the DNA does separate from the

membrane at termination and does stick to the membrane during thymine starvation.

A sequence of events leading to cell division has been proposed. Gradually, as results from various division mutants are gathered, modifications will certainly be made to the sequence, and the original proposals may be unrecognizable in the final result. Nevertheless such an attempt to distinguish various stages in the division process may be necessary for the design of further experiments.

CHAPTER 5: THE MIN-MUTATION

Introduction: Whereas the lon mutation appears under certain conditions to have difficulty initiating cell division, the min mutant might, at first sight, be considered to produce extra divisions. The min mutant (P678-54; MIN) was isolated by Adler et al (2, 4) as a mutant of E. coli P678 resistant to ionizing but not to ultraviolet irradiation. During normal logarithmic growth, these cells divide off small bodies from both poles. These minicells are about 10% of the volume of the normal cell and production of a minicell does not prevent normal division, although the two processes cannot occur simultaneously (3). They were originally thought to contain less than 10^{-3} times the amount of DNA present in a normal cell (2). It has since been claimed that if the nuclear material is near the tip of the cell at the time a minicell is divided off, a small fraction of the minicells do possess DNA due to the plane of division cutting through the DNA (157). Labelling with radioisotopes has also detected a small amount of DNA in minicells (89). This could be due to the same mechanism, however.

Although initially min was thought to be a single mutation close to but distinct from lon (3), it is now realized that two loci are involved, one (min A) in the pro C - pur E region (near lon) and the other (min B) in the pdx - pyr region (about half a minute on the Taylor map (155)) (126, K. Roozen, pers. commun.).

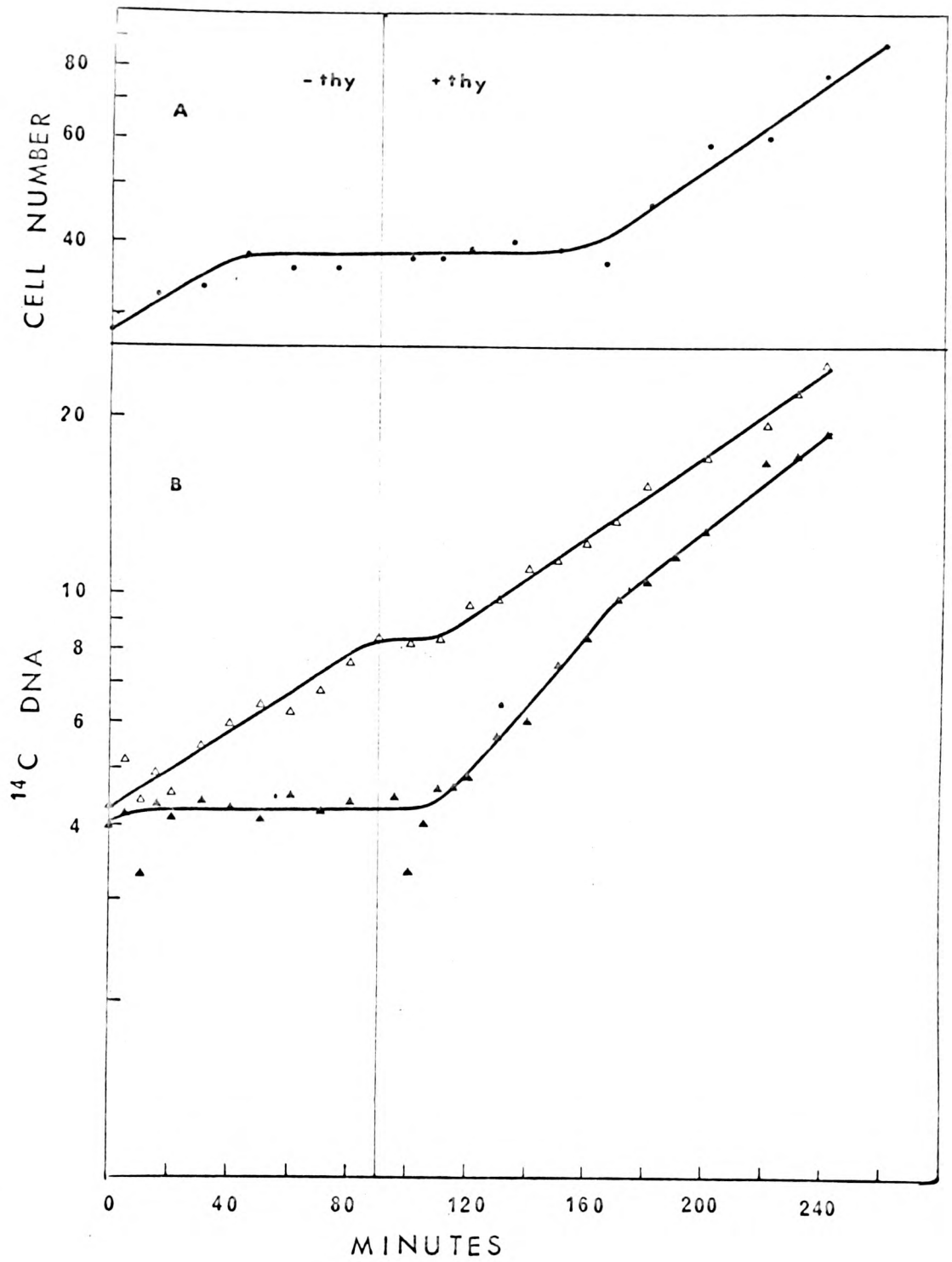
Minicells do not normally contain DNA (apart from the small amounts mentioned above), however, if the minicell strain is made F',

R^+ or col^+ (but not F^+ or P1 lysogenic), plasmid DNA is found in the minicells (80, 89, 125, 182, 183). Minicells from F^- minicell strains can act as recipients for DNA from F^+, F' or Hfr donors (24, 25). Minicells derived from F^+ or F' minicell strains cannot act as recipients for DNA even though they actually lack the F-pilus (24).

Because of the apparent close relationship between this mutation and the lon mutation (both affect cell division), and the close mapping of the min A and lon loci, it was decided to perform a parallel study on this strain to see if it yielded any further information on the division mechanism.

Effect of Thymine Starvation on the Cell: Since MIN shows a defect in the regulation of cell division, a check was made initially that DNA replication in this strain is normal. X806 min^- thy^- (MIN-T) was grown to log phase in minimal medium containing ^{14}C-thymine. Half the culture was starved of thymine and the other half reincubated in the presence of ^{14}C-thymine. After 90 min, ^{14}C-thymine was readded to the starved culture. Fig XXIIB shows the results obtained. The graph is very similar to that of Fig V. The prime difference is that the MIN strain shows a much less marked burst of DNA synthesis on readdition of thymine. The culture shows a slightly increased rate of DNA synthesis for the first 80 min after thymine has been readded and then settles down to a rate of synthesis similar to that of the unstarved culture. Since this strain was selected for resistance to ionizing irradiation, it is unlikely that the failure to restore the DNA content of the cells is due to many of the cells losing the ability to replicate their DNA.

Fig. XXII



DNA replication and cell division in thymine starved minicell-producing cells. A. Cells in minimal medium (50 µg/ml thymine) were filtered at time 0 and incubated in the absence of thymine. After 90 min, thymine was readded. B. Cells in minimal medium (10 µg/ml thymine) were treated as above except that pre- and post-starvation growth was in the presence of ^{14}C-thymine (0.2 µC/ml). ^{14}C-thymine uptake was followed as a measure of DNA synthesis.

The results of a similar experiment but using unlabelled thymine and following cell number is given in Fig.XXIIA. These results differ little from those of Fig. VIII. The slightly longer period of residual division and delay before resumption of division could be due to the slower growth of these organisms. This suggests that DNA replication and cell division after thymine starvation are normal in these cells.

Position of Divisions: It was suggested that minicells may be the result of misplaced divisions. If this were the case, they would likely be the extreme end of a spectrum of misplaced divisions. Log-phase cultures of MIN cells in minimal medium were inoculated onto minimal soft agar on a glass slide at 37°. Growth was observed and cells photographed as soon after division as possible. Fig. XXIII shows a plot of the total cell length (abscissa) against the distance of the division site from the nearest pole (ordinate). The lines drawn represent the position of median divisions and divisions at sites one quarter of the cell length. The results fall well along the median line, indicating a preference for the central division site which is also the oldest division site in the cell. The points which fall just above this line are due to the bias of the experiment since any errors in measurement are translated into one daughter cell being represented as too short. These results suggest that minicells do not represent extreme cases of misplaced cell division. If this were the case, there would be much more scatter of points.

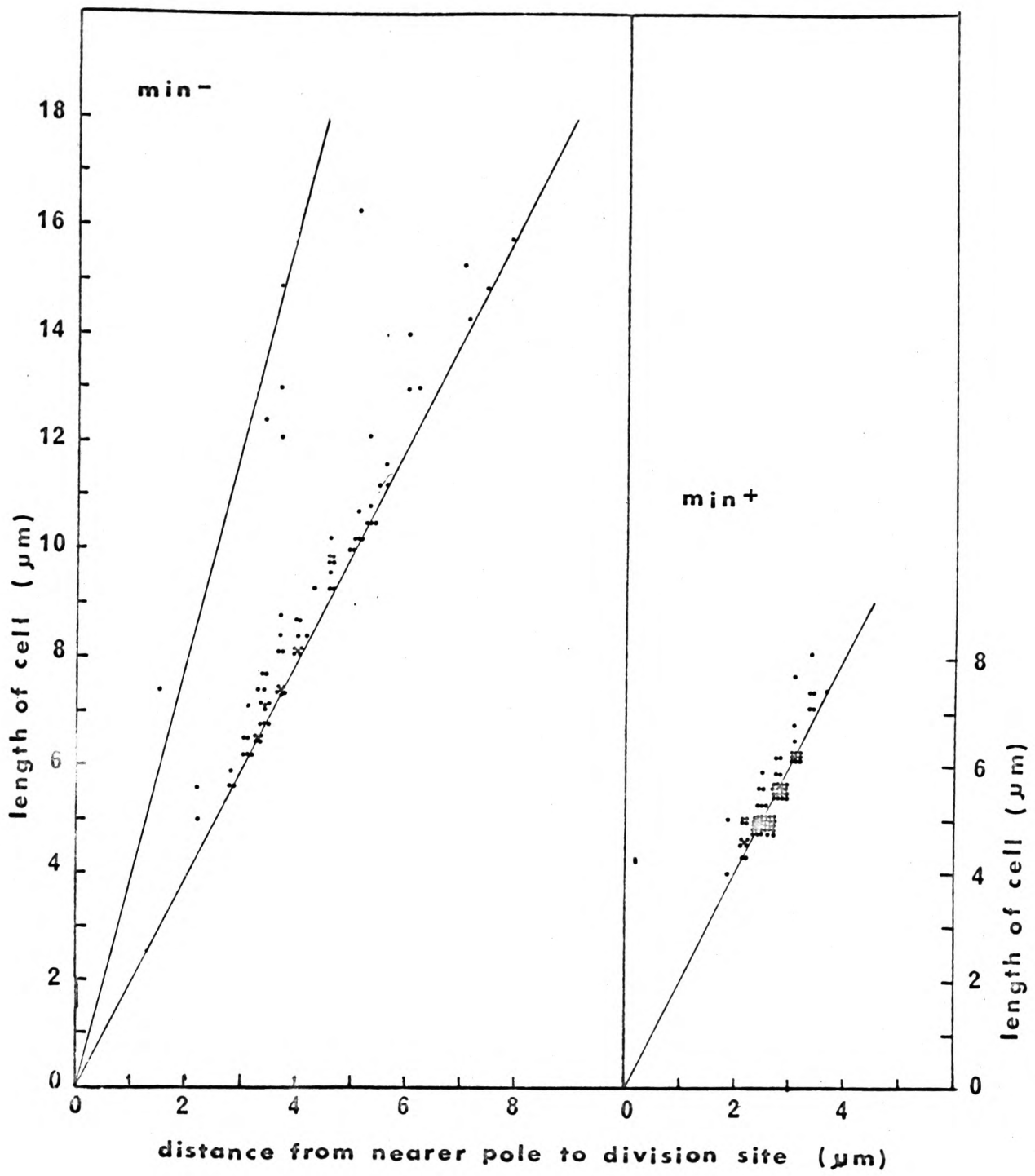
This idea had to be accepted with reservation since minicell production does not appear to bear any direct relationship to normal

division. That is, it does not seem to occur instead of division, nor does it occur specifically before or after division. Minicells can occur at either end of the cell irrespective of the position of the previous minicell or the previous division. Since Donachie & Begg (36) proposed that minimal grown cells grow from the end formed at the previous division, if minicells were formed instead of a division, they would be expected to occur at the newly formed end. This was not found to be necessarily the case.

However, the length of MIN cells is greater than that of wild-type cells. This is apparent under the microscope, and may also be seen by comparing the results of Fig. XXIII for $\underline{min}^+$ and $\underline{min}^-$ cells. The same experiment was performed using $\underline{min}^+$ cells. In this case, all the cells again fall on the median line and all the cells fall within a doubling of the cell length (from 1.9 - 3.7μ). *E. coli* $15T^-$ JG151 and B/r, both presumably $\underline{min}^+$, have been made to grow to lengths similar to those for $\underline{min}^-$ cells (36). In this case, there were three division sites/cell (at 25, 50 and 75% of the cell length). The 'newer' 25 and 75% sites, after the cell divides centrally, become the central sites of each of the two daughter cells. It thus appears that the sites take longer to develop in $\underline{min}^-$ cells and are not defective.

The measurements of cell lengths were liable to errors of up to 0.3μ (1mm on the enlarged image of the negative) and this will account for many of the points being off the line. The $\underline{min}^+$ cells

Fig. XXIII



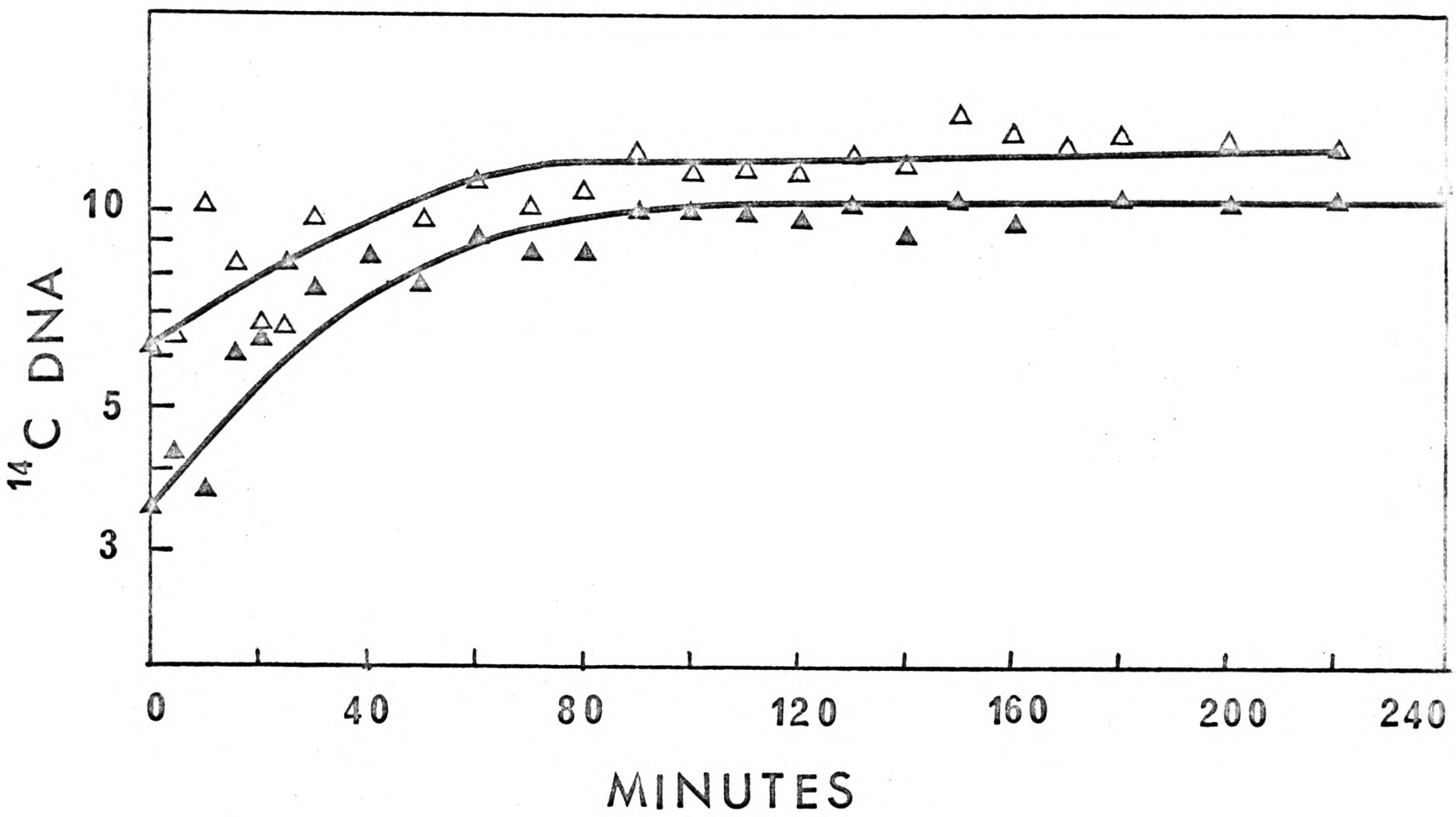
Division sites in untreated cells. Cells were grown to log-phase in minimal medium. They were then transferred to minimal soft agar and photographed when they divided. The lines drawn represent the positions of the division sites located at 50 and 25% of the cell length. Strains used: P678, min$^+$ and P678-54, min$^-$.

show a minimum size of 1.9μ very close to the value for the 'unit cell' found by Donachie & Begg for E.coli 15T$^-$ (36). The min$^-$ cells show a minimum length of 1.5μ. This difference is not likely to be significant.

DNA Synthesis During Amino Acid Starvation: The minicell strain was found to have a tendency to form long cells. This could be due to the cells possessing a long C-time. To test this possibility, MIN-T was starved of amino acids in the presence of ^{14}C-thymine, and its uptake followed. Fig. XXIV shows the results obtained for both min$^+$ and min$^-$ strains. The residual DNA synthesis lasted about 80 min. During this time, there was a 93% (min$^-$) and 126% (min$^+$) increase in the number of counts per minute of ^{14}C-thymine. Taking into account the slowing of the replication point caused by amino acid starvation (see Chapter 3), these results suggest that the C-time of these cultures is not abnormal. It is likely to be of the order of 40 min as found by Helmstetter and Cooper for B/r (28, 65). The increase in counts was about twice the increase expected (about 58%). This would indicate that some of the chromosomes were multiforked, although, since the cells were growing in 30μg/ml thymine, this was not expected.

Acrylamide gel electrophoresis: Since minicells are enucleate cells, this suggested that if the DNA is connected to a specific site on the membrane, this.site might be lacking from minicells, and the profile on gel electrophoresis would be different. For reasons of economy, it was necessary to use $^{35}SO_4$ instead of a ^{14}C-labelled

Fig. XXIV



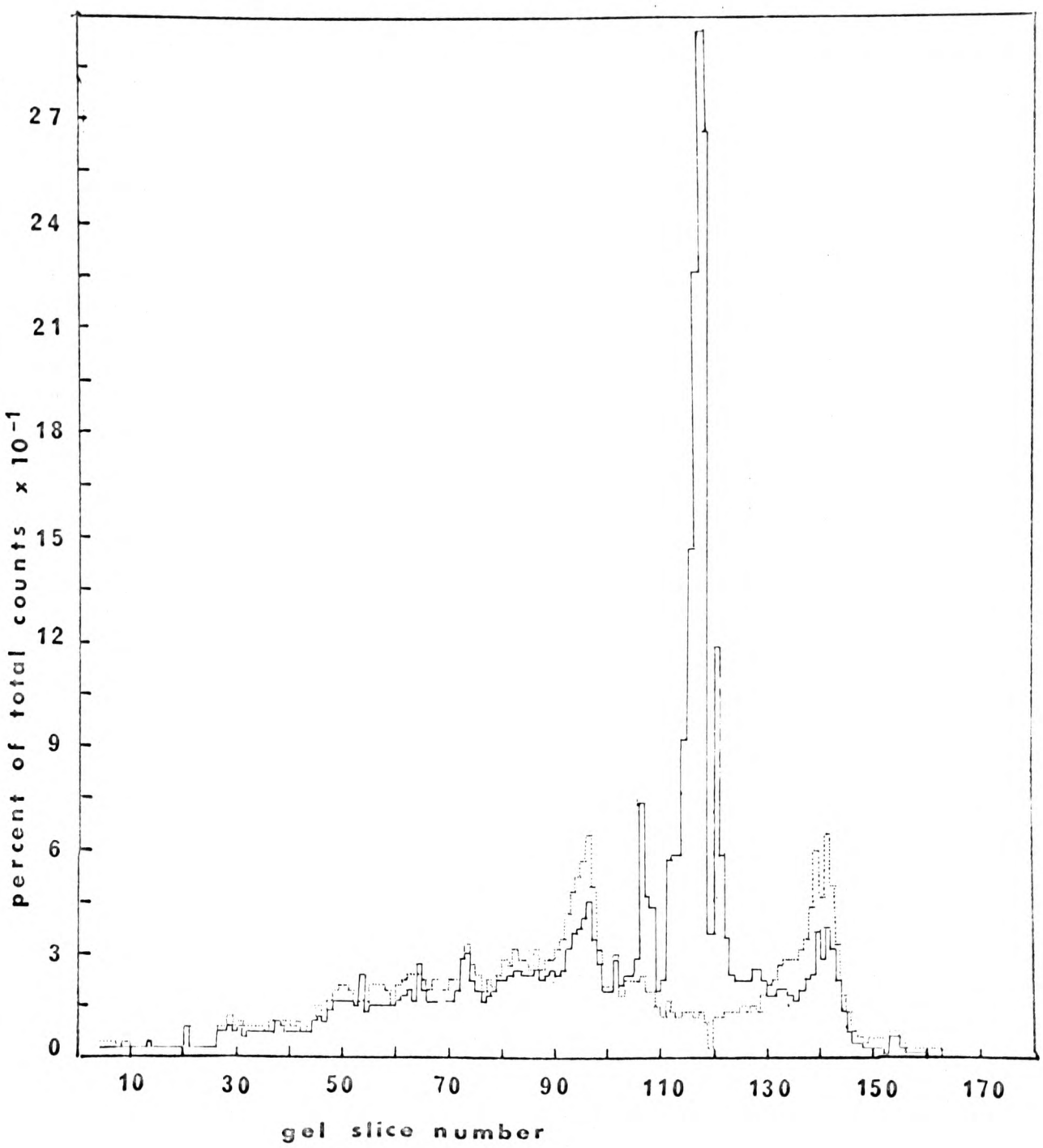
DNA synthesis during amino acid starvation. Cells were grown for 3 mass doubling times in the presence of ^{14}C-thymine (30 µg/ml, 0.6 µC/ml). At time 0, they were filtered and resuspended in the absence of amino acids, and the uptake of ^{14}C-thymine followed. X806 (△), P678-T (▲).

amino acid. ^{3}H-methionine was used as the other label. The cells from which the minicells separate will be called 'mother cells'. To check if the isotopes label the proteins in the same manner , comparison was made of envelope preparations from ^{3}H and ^{35}S-labelled mother cells. The results are seen in Fig. XXV. The ^{35}S profile compares well with that of the lon cells (see Fig. XIX & XX). In this strain, there is a large fast moving peak of equal height to that of the peak found around gel slice number 90. Apart from this, there appears to be little difference between the profiles for min and lon strains. The ^{3}H labelled cells show an extra very large set of peaks around gel slices 115-120. The reason for this large peak is not known. One possibility is that the ^{3}H-label was removed from the methionine and added to other amino acids. This peak, presumably a low sulphur containing protein, does appear in the lon profile, but as a much smaller peak. With Fig. XXV, it would seem that this molecule has become heavily labelled with ^{3}H.

Because of these results, it was considered more helpful to compare the ^{3}H-labelled minicells with the ^{3}H-labelled mother cells, and similarly for the ^{35}S-labelled cells. These results are seen in Fig. XXVI and XXVII. Since the minicells and mother cells are from different preparations and different gels, they do not correspond exactly. In order to facilitate their comparison, the minicell profile has been moved one slice to the left (Fig. XXVI) or two slices to the right (Fig XXVII).

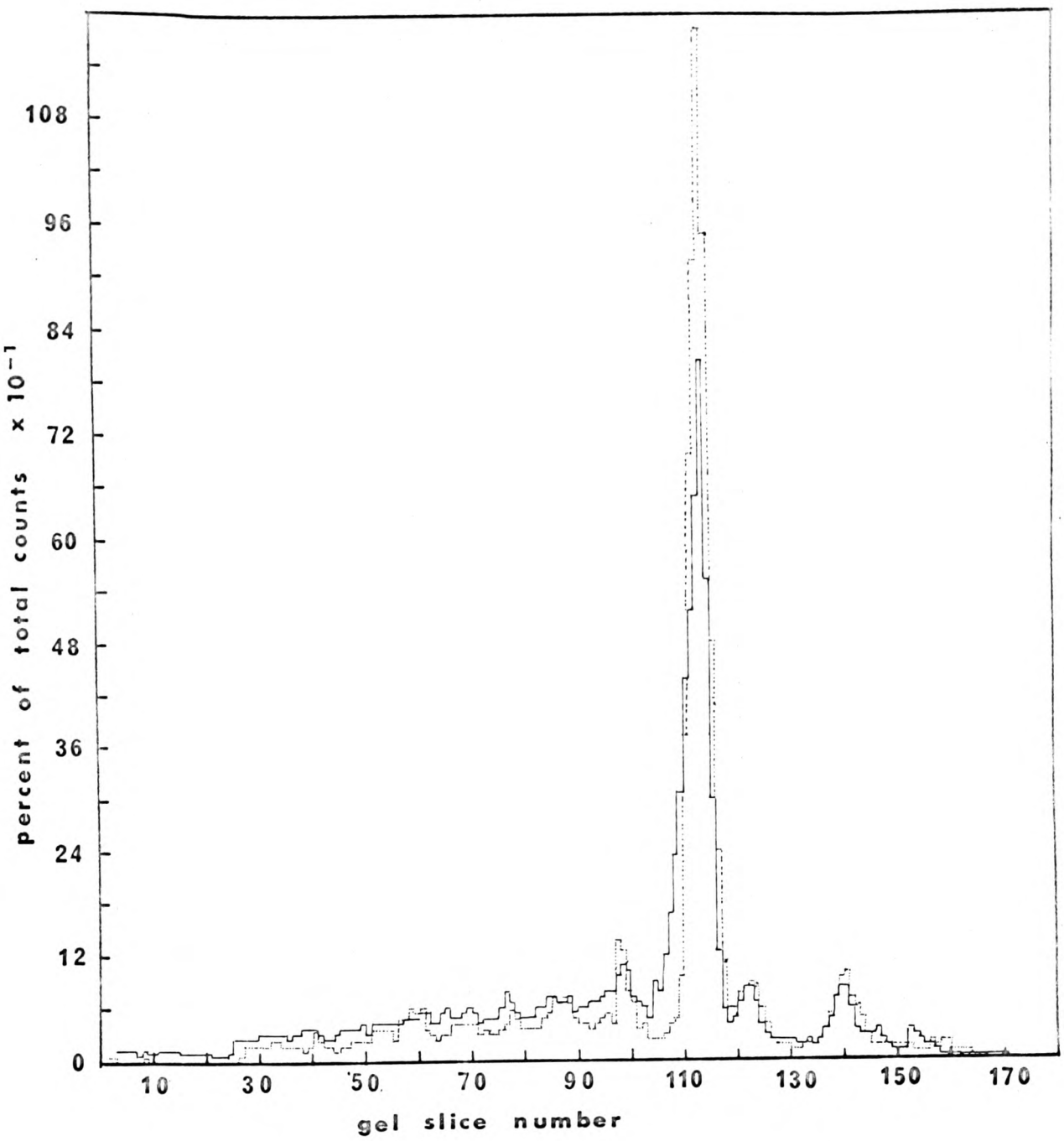
The set of peaks centred on slice 115 seen in Fig. XXV appears as a single peak in Fig. XXVI. I suspect that the latter, being

Fig. XXV



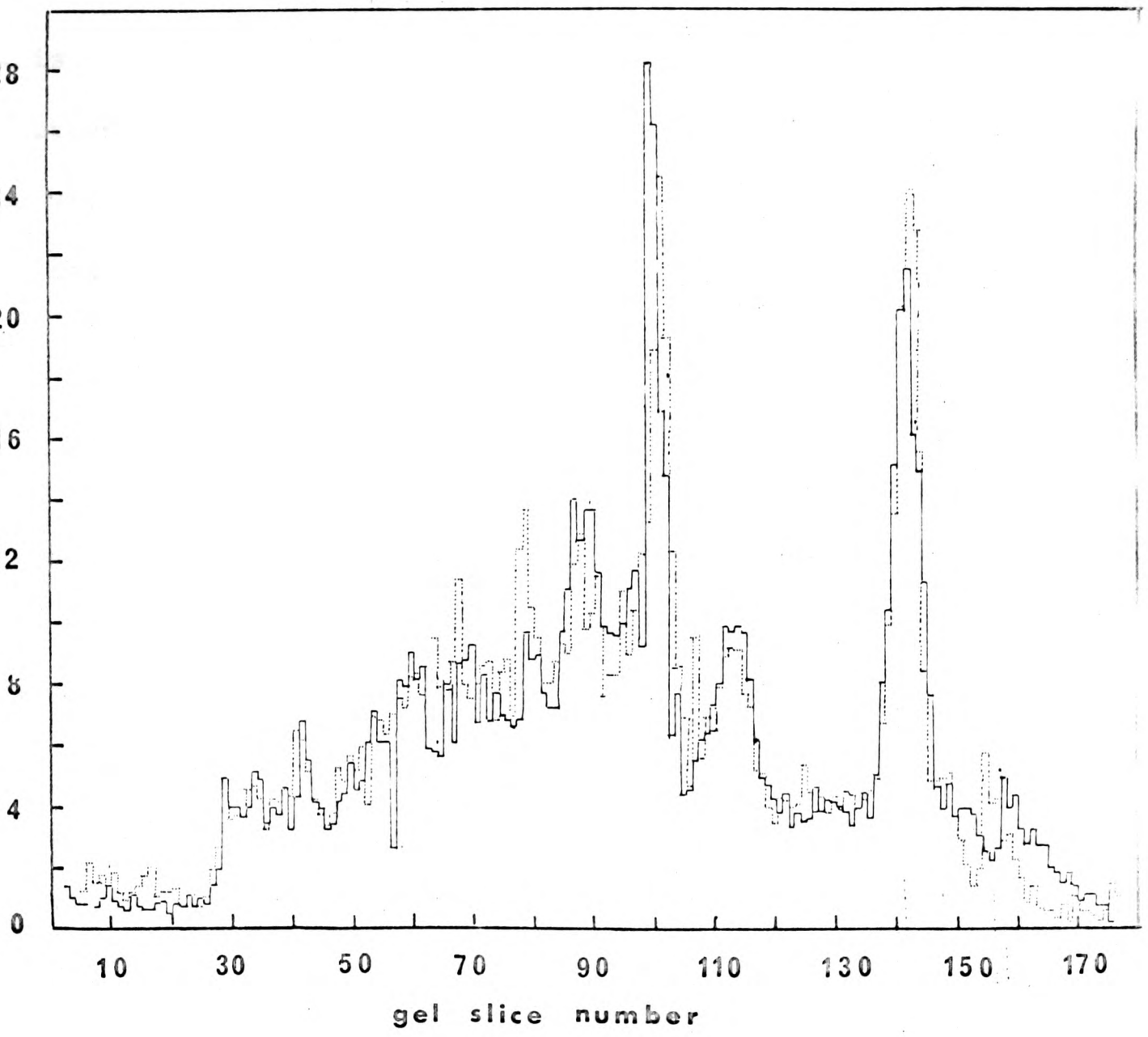
As Fig. XIX. 'Mother cells' (see text) of P678-54, min- labelled with ^{3}H-methionine (solid line) or $^{35}SO_4$ (dotted line).

Fig. XXVI



As Fig. XIX. 'Mother cells' (solid line) and minicells (dotted line) of P678-54, min⁻ labelled with ^{3}H-methionine. The minicell profile has been moved 1 slice to the left.

Fig. XXVII



As Fig. XIX. 'Mother cells' (solid line) and minicells (dotted line) of P678-54, min⁻ labelled with $^{35}SO_4$. The minicell profile has been moved 2 slices to the right.

a fresher preparation (the cells for Fig. XXV were maintained after washing at -50° until used) is the more normal picture. This peak is higher in minicells than in mother cells, but there is no indication of this difference in the smaller corresponding peak found with sulphur labelling (Fig XXVII). The size of this peak means that in order to plot the results all on the same scale, much of the minor variations in peak height disappear (as do, of course, any very small peaks). However, this does emphasize any significant peaks which are present. There are a few minor differences in the smaller peaks near the top of the gel, however, these do not appear with sulphur labelling and so are likely to be artifacts. Being a larger scale, Fig. XXVII shows all chance variations in the number of counts in a gel slice and the graph is less smooth than Fig. XXVI. However, there is good correspondence between mini-cell and 'mother cell' for all peaks.

From the results of Schnaitman (137), it appears that these protein profiles contain much cell wall material. As mentioned before, the peak at gel slice 100 (slice 90 in lon) appears to correspond to Schnaitman's peak 16 and the new peak at gel slice 115 is likely to be his peak 20 or 21, which appear to consist of both cell wall and cytoplasmic membrane proteins. Separating the two fractions appears to be essential for accurate study of the bacterial envelope. No differences were unequivocally demonstrated between the cell envelopes of minicells and the mother cells from which they divide.

Conclusions: Minicells as small enucleate cells are not unique. Hirota et al (179) described two mutations of E. coli T46 and divA which together gave the same effect as min. T46 is a temperature sensitive mutation causing inhibition of DNA synthesis at 40°. As a consequence, cell division also ceases. DivA is a second mutation expressed only in the presence of T46 which allows division to occur in the absence of DNA synthesis, so that enucleate cells are divided off the filament. These enucleate cells are normal in size however, whereas minicells are smaller than normal. Inouye (180) described another mutation of E. coli causing cell division to continue even when DNA synthesis has been inhibited by a shift to 41°. To explain his results, Inouye hypothesized a factor M which normally is bound to the DNA replication apparatus and also inhibits septum formation. Completion of DNA replication produces factor I which suppresses M allowing division to occur. He suggested that at 41°, M separates from the DNA replication apparatus stopping DNA synthesis but allowing 'constitutive' septum formation.

Donachie, Martin & Begg (submitted for publication) found that B. subtilis does not possess this control of division, so that division continues even in the absence of DNA replication, again dividing off normal sized enucleate cells.

These experiments were an attempt to see if minicell-producing strains are abnormal in any of a few characteristics. As far as could be seen, they were not. DNA replication was found to be normal, both with respect to its C-time (Fig. XXIV) and its kinetics

after inhibition by thymine starvation (Fig. XXII). Similarly, the pattern of cell division of MIN cells after thymine starvation is much like that of *lon*$^+$ cells (Fig. VIII, XXII), nor was there any indication from observation of the minicell producing cells after thymine starvation of any increased or decreased rate of minicell production. The position of the division site of the minicell-producing cells was normal, indicating that minicells are not the product of abnormal divisions. Observation of minicell-producing cells revealed that they are larger than wild-type cells, but this is not due to a long C-time as might have been the cause. It is possible that minicells are the cells' attempts to correct their abnormal length. It would be a great coincidence if the increased size of the *min*$^-$ cells was unrelated to their mini-cell producing property, since both demonstrate effects on the cell division mechanism.

It was not possible to identify a membrane protein likely to be the DNA attachment site. This could be because there is no unique site for its attachment, but rather a modification of normal cell membrane. It is also possible that, due to the mixture of wall and membrane proteins in the preparation, the small amount of protein present in mother cells but not in minicells was missed. In view of the discrepancies found with the pair of labels used, ^3H and ^{14}C-leucine (as used for the *lon* strains) would be preferable as the protein labels.

Two theories principally were examined. The first was that minicells are the result of misplaced divisions. This was found not

to be the case. The position of the other division sites is normal, and minicell production appeared to bear no relationship to division with respect to timing or the end of the cell at which the minicell appeared. The other theory, also found not to be the case, was that minicells possess an abnormally long C-time. Although the amount of residual DNA synthesis is greater than expected, the C-time was normal and the same for *min*$^+$ and *min*$^-$ cells.

Two very simple explanations for minicell production would be either that two division sites are formed close to each other, or that division sites are formed simultaneously at both ends of the cell. Both of these give very definite predictions as to the end of the cell at which minicells appear. Since they appear at either end apparently randomly, neither of these can be the mechanism of action of the *min*-mutation. Rather the mutation is likely to be in a control mechanism, whereby a second division is initiated in error, but not at any one particular place. Not being linked to the growth site, it does not need to follow the pattern of Donachie & Begg (36) and occur at the end where growth is occurring. However, minicell production is prevented by inhibiting DNA synthesis (23, my own results) indicating that the stage of division in which the error occurs is dependent on termination of chromosome replication. Adler *et al* (3) made a *lon*$^-$ *min*$^-$ mutant of *E. coli* and found that in their observations, filaments never produced minicells. This is further indication that minicell production is under exactly the same control as normal cell division. It would be interesting to examine the position of the minicells in filaments which have started dividing after thymine starvation, since this might give an indication of what

determines the position of the minicell. It may be that these cells produce excess of an activator of division (? produced at termination of DNA synthesis in the previous cell cycle) as hypothesized by Inouye (180) and Donachie (pers. commun.). This would imply that septum formation begins before completion of the previous division.

Another examination of the membrane proteins of minicells and mother cells is planned, this time with the technique improved. In view of the very small difference that would be expected, all wall proteins must be removed.

Discovering the effect on the cell of the min mutation would give an indication of one of the control mechanisms for cell division. So far, most work with minicells has been as recipients for DNA from other cells. However, indications are that although minicells arise from errors in initiation of division, the actual mechanism of division resulting in the minicell is normal. Studies on minicell production could therefore complement cell division studies.

REFERENCES

1. Adler, H.I., W.D.Fisher, A.A.Hardigree & G.E.Stapleton, (1966), J. Bact., 91: 737-742.
2. Adler, H.I., W.D.Fisher, A.Cohen & A.A.Hardigree, (1967), Proc. US Nat. Acad. Sci., 57: 321-326.
3. Adler, H.I., W.D.Fisher & A.A.Hardigree,(1969), Trans. N.Y. Acad. Sci., Ser. II, 31: 1059-1070.
4. Adler, H.I., W.D.Fisher & G.E.Stapleton, (1966), Science, 154 : 417.
5. Adler, H.I. & A.A.Hardigree, (1964), J. Bact., 87: 720-726.
6. Adler, H.I. & A.A.Hardigree, (1964), Radiation Res., 22: 165.
7. Adler, H.I. & A.A.Hardigree, (1965), J.Bact., 90: 223-226.
8. Adler, H.I. & A.A.Hardigree, (1965), Radiation Res., 25: 92-102.
9. Anderson, E.H., (1951), J. Bact., 61: 389-394.
10. Baker, J.R., (1948), Quart. J. Micr. Sci., 89: 103-125.
11. Baker, J.R., (1949), Quart. J. Micr. Sci., 90: 87-108.
12. Baker, J.R., (1952), Quart. J. Micr. Sci., 93: 157-190.
13. Bazill, G.W., (1967), Nature, 216: 346-349.
14. Billen, D., (1969), J. Bact., 97: 1169-1175.
15. Billen, D., R.Hewitt & E.Frampton, (1966), Radiation Res., Suppl. 6: 212-214.
16. Bisset, K.A., (1950), The cytology and life-history of bacteria, Edinburgh, E. & S. Livingstone.
17. Bisset, K.A., (1956), Symp. Soc. Gen. Microbiol., 6: 1-18.
18. Cairns, J., (1963), Cold Sp. Harb. Symp. Quant. Biol., 28:43-46.

19. Cameron, G.R., (1952), Pathology of the cell, Edinburgh, Oliver & Boyd.

20. Caro, L.G. & C.M.Berg, (1968), Cold Sp. Harb. Symp. Quant. Biol., 33: 559-573.

21. Chai, N. & K.G. Lark, (1967), J. Bact., 94: 415-421.

22. Clark, A.J. & A.D.Margulies, (1965), Proc. US Nat. Acad. Sci., 53: 451-459.

23. Clark, D.J., (1968), Cold Sp. Harb. Symp. Quant. Biol., 33: 823-838.

24. Cohen, A., W.D.Fisher, R. Curtiss III & H.I.Adler, (1968), Cold Sp. Harb. Symp. Quant. Biol., 33: 635-641.

25. Cohen, A., W.D.Fisher, R. Curtiss III & H.I.Adler, (1968), Proc. US Nat. Acad. Sci., 61: 61-68.

26. Cohen, S.S. & H.D.Barner, (1954), Proc. US Nat. Acad. Sci., 40: 885-893.

27. Cole, R.M., (1965), Bacteriol. Rev., 29: 326-344.

28. Cooper, S. & C.E.Helmstetter, (1968), J. Mol. Biol., 31: 519-540.

29. Cuzin, F. & F. Jacob, (1967), Ann. Inst. Past., 112: 529-546.

30. Davies, J., (1966), Cold Sp. Harb. Symp. Quant. Biol., 31: 665-670.

31. Deering, R.A., (1958), J. Bact., 76: 123-130.

32. Deering, R.A. & R.B.Setlow, (1957), Science, 126: 397-398.

33. Deering, R.A. & R.B.Setlow, (1963), Biochim. Biophys. Acta, 68: 526-534.

34. Donachie, W.D., (1968), Nature; 219: 1077-1079.

35. Donachie, W.D., (1969), J. Bact., 100: 260-268.

36. Donachie, W.D. & K.J.Begg, (1970), Nature, 227: 1220-1224.

37. Donachie, W.D. & D.G.Hobbs, (1967), Biochem. Biophys. Res. Commun., 29: 172-177.

38. Donachie, W.D., D.G.Hobbs & M.Masters, (1968), Nature, 219: 1079-1080.

39. Donch, J., Y.S. Chung & J. Greenberg, (1969), Genetics, 61: 363-370.

40. Donch, J., M.H.L. Green & J. Greenberg, (1968), J.Bact., 96: 1704-1710.

41. Donch, J. & J. Greenberg, (1968), J. Bact., 95: 1555-1559.

42. Donch, J. & J. Greenberg, (1968), Molec. Gen. Genet., 103: 105-115.

43. Doudney, C.O., (1968), Curr. Topics Microbiol. Immunol., 46: 116-175.

44. Driedger, A.A., (1970), Can. J. Microbiol., 16: 881-882.

45. Eaton, N.R., (1962), J. Bact., 83: 1359-1360.

46. Ellar, D.J., D.G.Landgren & R.A.Slepecky, (1967), J. Bact., 94: 1189-1205.

47. Emmerson, P.T., (1968), Genetics, 60: 19-30.

48. Endo, H., K. Ayabe, K.Amaho & K. Takeya, (1965), Virology, 25: 469-471.

49. Errera, M., (1954), Br. J. Radiol., 27: 76-80.

50. Fielding, P. & C.F.Fox, (1970), Biochem. Biophys. Res. Commun., 41: 157-162.

51. Fisher, W.D., H.I.Adler, F.W.Shull Jr. & A. Cohen, (1969), J. Bact., 97: 500-505.

52. Fitz-James, P.C., (1960), J. Biophys. Biochem. Cytol., 8: 507-528.

53. Fitz-James, P.C., (1965), Bacteriol. Rev., 29: 293-298.

54. Freifelder, D., (1969), J. Mol. Biol., 45:1-7.

55. Fuhs, G.W., (1965), Bacteriol. Rev., 29: 277-293.

56. Fuchs, E. & P. Hanawalt, (1970), J. Mol. Biol., 52 301-322.

57. Ganesan, A.T. & J. Lederberg, (1965), Biochem. Biophys. Res. Commun., 18: 824-835.

58. Ganesan, A.K., & K.C. Smith, (1968), Cold Sp. Harb. Symp. Quant. Biol., 33: 235-242.

59. Gates, F.L., (1933), Science, 77: 350.

60. Gorini, L. & E. Kataja, (1965), Biochem. Biophys. Res. Commun., 18 656-663.

61. Green, M.H.L., J. Donch & J. Greenberg, (1969), Mutation Res., 8: 409-411.

62. Greenberg, J., (1965), Mutation Res., 2: 304-311.

63. Grula, E.A. & M.M. Grula (1962), J. Bact., 83: 981-988.

64. Haynes, R.H., (1966), Radiation Res., Suppl. 6: 1-29.

65. Helmstetter, C.E. & S. Cooper, (1968), J. Mol. Biol., 31: 507-518.

66. Helmstetter, C., S. Cooper, O. Pierucci & E. Revelas, (1968), Cold Sp. Harb. Symp. Quant. Biol., 33: 809-822.

67. Hewitt, R. & D. Billen, (1965), J. Mol. Biol., 13: 40-53.

68. Highton, P.J., (1969), J. Ultrastructure Res., 26: 130-147.

69. Highton, P.J., (1970), J. Ultrastructure Res., 31: 260-271.

70. Hoffman, H. & M.E.Frank, (1965), J. Bact., 89: 212-216.

71. Howard-Flanders, P., (1964), J. Cellular Comp. Physiol., 64: Suppl. 51-68.

72. Howard-Flanders, P., (1968), Adv. Biological Medical Physics, 12: 299-317.

73. Howard-Flanders, P., R.P.Boyce, E. Simson & L. Theriot, (1962), Proc. US Nat. Acad. Sci., 48: 2109-2115.

74. Howard-Flanders, P., R.P. Boyce, & L. Theriot, (1966), Genetics, 53: 1119-1136.

75. Howard-Flanders, P., E. Simson & L. Theriot, (1964), Genetics, 49: 237-246.

76. Howard-Flanders, P., E. Simson & L. Theriot (1964), Mutation Res., 1: 219-226.

77. Howard-Flanders, P. & L. Theriot, (1966), Genetics, 53: 1137-1150.

78. Inouye, M. & J.P. Guthrie, (1969), Proc. US Nat. Acad. Sci., 64: 957-961.

79. Inouye, M. & A.B.Pardee, (1970), J. Biol. Chem., 245:5813-5819.

80. Inselburg, J., (1970), J. Bact., 102: 642-647.

81. Ivarie, R.D. & J.J.Pene, (1970), J. Bact., 104: 839-850.

82. Jacob, F., S. Brenner & F. Cuzin, (1963), Cold Sp. Harb. Symp. Quant. Biol., 28: 329-348.

83. Jacob, F., A. Ryter & F. Cuzin, (1966), Proc. Roy. Soc., Ser. B, 164: 267-278.

84. Jagger, J., (1960), Radiation Res., 13: 521-539.

85. Kalle, G.P., & P.P. Sivasubramanian, (1967), Radiation Res., 30: 71-80.

86. Kanazir, D., (1954), Biochim. Biophys. Acta, 13: 589-590.

87. Kanazir, D. & M. Errera, (1954), Biochim. Biophys. Acta, 14: 62-66.

88. Kantor, G.J. & R.A.Deering, (1968), J. Bact., 95: 520-530.

89. Kass, L.R. & M.B.Yarmolinsky, (1970), Proc. US Nat. Acad. Sci., 66: 815-822.

90. Kelner, A., (1953), J. Bact., 65: 252-262.

91. Kirby, E.P., F. Jacob & D.A.Goldthwait, (1967), Proc. US Nat. Acad. Sci., 58: 1903-1910.

92. Knaysi, G., (1946), Elements of bacterial cytology, Ithaca, N.Y., Comstock.

93. Lark, K.G. & R. Pritchard, (1963), J. Cell Biol., 19: 43A.

94. Lark, K.G. & H. Renger, (1969), J. Mol. Biol., 42: 221-235.

95. Lieberman, M.M. & A. Markovitz, (1970), J. Bact., 101: 965-972.

96. Markovitz, A., (1964), Proc. US Nat. Acad. Sci., 51:239-246.

97. Markovitz, A. & B. Baker, (1967), J. Bact., 94: 388-395.

98. Markovitz, A. & N. Rosenbaum, (1965), Proc. US Nat. Acad. Sci., 54: 1084-1091.

99. Markovitz, A., N. Rosenbaum & B.Baker, (1968), J. Bact., 96: 221-226.

100. Martin, D.T.M., (1970), Ph.D. Thesis, Edinburgh

101. Mennigmann, H-D, (1964), Biochem. Biophys. Res. Commun., 16: 373-378.

102. Mennigmann, H-D, (1965), J. gen. Microbiol., 41: 151-154.

103. Mennigmann, H-D & W. Szybalski, (1962), Biochem. Biophys. Res. Commun., 9: 398-404.

104. Mukai, F.H., (1960), J. gen. Microbiol., 23: 539-551.

105. Nagel de Zwaig, R. & S.E.Luria, (1967), J. Bact., 94: 1112-1123.

106. Nakamura, H., (1968), J. Bact., 96: 987-996.

107. Neuhard, J., (1966), Biochim. Biophys. Acta, 129:104-115.

108. Nomura, M. & C. Witten, (1967), J. Bact., 94: 1093-1111.

109. Ornstein, L., (1964), Ann. N.Y. Acad. Sci., 121: 321-349.

110. Pauling, C. & P. Hanawalt, (1965), Proc. US Nat. Acad. Sci., 54: 1728-1735.

111. Pelczar, M.J. & R.D.Reid, (1958), Microbiology, N.Y., McGraw Hill.

112. Picken, L., (1960), The organization of cells and other organisms, Oxford, Clarendon Press.

113. Pierucci, O. & C.E.Helmstetter, (1969), Fed. Proc., 28: 1755-1760.

114. Pontefract, R.D., G. Bergeron & F.S.Thatcher, (1969), J. Bact., 97: 367-375.

115. Pritchard, R.H., P.T. Barth & J. Collins, (1969), Symp. Soc. Gen. Microbiol., 19: 263-297.

116. Pritchard, R.H. & K.G.Lark (1964), J. Mol. Biol., 9: 288-307.

117. Pritchard, R.H. & A. Zaritsky, (1970), Nature, 226: 126-131.

118. Radman, M., L. Cordone, D. Krsmanovic-Simic & M. Errera, (1970), J. Mol. Biol., 49: 203-212.

119. Raymond, S., (1964), Ann. N.Y. Acad. Sci., 121: 350-365.

120. Roberts, R.B., P.H.Abelson, D.B.Cowie, E.T.Bolton & R.J.Britten, (1955), Studies of biosynthesis in Escherichia coli, Washington, D.C., Carnegie Institution of Washington Publication 607.

121. Roberts, R.B., & E. Aldous, (1949), J. Bact., 57: 363-375.

122. Robinow, C.F., (1942), Proc. Roy. Soc., Ser. B, 130: 299-324.

123. Robinow, C.F., (1944), J. Hyg., 43: 413-423.

124. Rogers, H.J., (1970), Bacteriol. Rev., 34: 194-214.

125. Roozen, K.J., R.G.Fenwick Jr., S.Levy & R.Curtiss III, (1970), Genetics, Suppl., 64: s54.

126. Roozen, K. & R. Curtiss III, (1969), Abstr. 4607A, Oak Ridge National Laboratory.

127. Rörsch, A., A. Edelman, C. van der Kamp & J.A.Cohen, (1962), Biochim. Biophys. Acta., 61: 278-289.

128. Rupp, W.D. & P. Howard-Flanders, (1968), J. Mol. Biol., 31: 291-304.

129. Ryan, F.J., P. Fried & F. Mukai, (1955), Biochim. Biophys. Acta, 18: 131.

130. Ryter, A., (1968), Bacteriol. Rev., 32: 39-54.

131. Ryter, A. & F. Jacob, (1964), Ann. Inst. Pasteur, 107: 384-400.

132. Ryter, A. & F. Jacob, (1966), Ann. Inst. Pasteur, 110: 801-812.

133. Salton, M.R.J., (1964), The bacterial cell wall, London, Elsevier.

134. Samson, A.C.R. & I.B.Holland, (1970), FEBS Letters, 11: 33-36.

135. Sandoval, H.K., H.C.Reilly & B, Tandler, (1965), Nature, 205: 522-523.

136. Schnaitman, C.A., (1970), J. Bact., 104: 882-889.

137. Schnaitman, C.A., (1970), J. Bact., 104: 890-901.

138. Schnaitman, C., (1970), J. Bact., 104: 1404-1405

139. Setlow, J.K., (1966), Radiation Res., Suppl. 6: 141-155.

140. Setlow, R.B. & W.L.Carrier, (1964), Proc. US Nat. Acad. Sci., 51: 226-231.

141. Setlow, R.B. & W.L.Carrier, (1966), J. Mol. Biol., 17: 237-254.

142. Setlow, R.B. & J.K.Setlow, (1962), Proc. US Nat. Acad. Sci., 48: 1250-1257.

143. Setlow, R.B., P.A.Swenson & W.L.Carrier, (1963), Science, 142: 1464-1466.

144. Skavronskaya, A.G. & G.B. Smirnov, (1969), Mutation Res., 8: 647-650.

145. Smith, D.T., N.F. Conant & J.R.Overman, (1964), Zinsser Microbiology, 13th ed., N.Y., Appleton-Century-Crofts.

146. Smith, D.W. & P.C.Hanawalt, (1967), Biochim. Biophys. Acta, 149: 519-531.

147. Smith, H.S. & A.B.Pardee (1970), J. Bact., 101: 901-909.

148. Smith, I., (1968), Acrylamide gel electrophoresis, in Chromatographic and electrophoretic techniques,vol II, Smith, I., (ed.), London, William Heinemann.

149. Smith, K., (1966), Radiation Res., Suppl. 6: 54-79.

150. Stacey, K.A. & E. Simson, (1965), J. Bact., 90: 554-555.

151. Stárka, J. & J. Moravová, (1967), Folia Microbiol., 12: 240-247.

152. Steed, P. & R.G.E.Murray, (1966), Can. J. Microbiol., 12: 263-270.

153. Sueoka, N. & W.G.Quinn, (1968), Cold Sp. Harb. Symp. Quant. Biol., 33: 695-705.

154. Swenson, P.A. & R.B.Setlow, (1966), J. Mol. Biol., 15: 201-219.

155. Taylor, A.L., (1970), Bacteriol. Rev., 34: 155-175.

156. Tremblay, G.Y., M.J. Daniels & M. Schaechter, (1969), J. Mol. Biol., 40: 65-76.

157. Tudor, J., T. Hashimoto & S.F.Conti, (1969), J. Bact., 98: 298-299.

158. Uretz, R.B. & A. Markovitz, (1969), J. Bact., 100: 1118-1120.

159. van de Putte, P., C.A. van Sluis, J. van Dillewijn & A. Rörsch, (1965), Mutation Res., 2: 97-110.

160. van de Putte, P., C. Westenbroek & A. Rörsch, (1963), Biochim. Biophys. Acta, 76: 247-256.

161. Vogel, H.J. & D.M.Bonner, (1956), J. Biol. Chem., 218: 97-106.

162. Walker, J.R., (1969), J. Bact., 99: 713-719.

163. Walker, J.R., (1970), J. Bact., 104: 1391-1392.

164. Walker, J.R. & A.B.Pardee, (1967), J. Bact., 93: 107-114.

165. Walker, J.R. & A.B.Pardee, (1968), J. Bact., 95: 123-131.

166. Walker, J.R. & J.A.Smith, (1970), Mol. Gen. Genet., 108: 249-257.

167. Ward, C.B. & D.A.Glaser, (1969), Proc. US Nat. Acad. Sci., 64: 905-912.

168. Ward, C.B. & D.A.Glaser, (1970), Proc. US Nat. Acad. Sci., 67: 255-263.

169. Ward, C.B., M.W.Hane & D.A.Glaser, (1970), Proc. US Nat. Acad. Sci., 66: 365-369.

170. Weinbaum, G., (1966), J. gen. Microbiol., 42: 83-92.

171. Weinbaum, G., D.A. Fischman & S. Okuda, (1970), J. Cell. Biol., 45: 493-508.

172. Wise, E.M. & J.T. Park, (1965), Proc. US Nat. Acad. Sci., 54: 75-81.

173. Witkin, E.M., (1946), Proc. US Nat. Acad. Sci., 32: 59-68.

174. Witkin, E.M., (1964), Mutation Res., 1: 22-36.

175. Witkin, E.M., (1966), Radiation Res., Suppl. 6: 26

176. Witkin, E.M., (1967), Proc. US Nat. Acad. Sci., 57: 1275-1279.

177. Witkin, E.M., (1969), Ann. Rev. Genet., 3: 525-552.

ADDITIONAL REFERENCES

178. Bird, R.E. & K.G. Lark, (1970), J. Mol. Biol., 49: 343-366.

179 Hirota, Y., F. Jacob, A. Ryter, G. Buttin & T. Nakai, (1968), J. Mol. Biol., 35: 175-192.

180. Inouye, M., (1969), J. Bact., 99: 842-850.

181. Inouye, M. & A.B.Pardee, (1970), J. Bact., 101: 770-776.

182. Inselberg, J., (1971), J. Bact., 105: 620-628.

183. Levy, S.B. & P. Norman, (1970), Nature, 227: 606-607.

184. Martin, E.L. & R.A. MacLeod, (1971), J. Bact., 105: 1160-1167.

185. Rosenberg, B.H. & L.F.Cavalieri, (1968), Cold Sp. Harb. Symp. Quant. Biol., 33: 65-72.

ACKNOWLEDGEMENTS

I would like to express my thanks to Prof. Hayes for the effort he put into finding financial support for me, and to the Wellcome Foundation for providing that support in the form of a Research Training Fellowship.

I must thank Willie for his advice, his enthusiasm and his ready availability during the conduct of this work.

Ulrich Loening was kind enough to teach me the gel electrophoresis technique and made his facilities freely available to me.

The number of people in the Departments of Molecular Biology and Zoology who have helped me at various times are too numerous to mention individually, but I thank them all.

I also want to express my appreciation to Rod for printing the photographs and to Rosemary for the excellent typing of this thesis.

And finally Marilynn, for the inspiration, enthusiasm, help, the raison d'être, thanks are not enough.

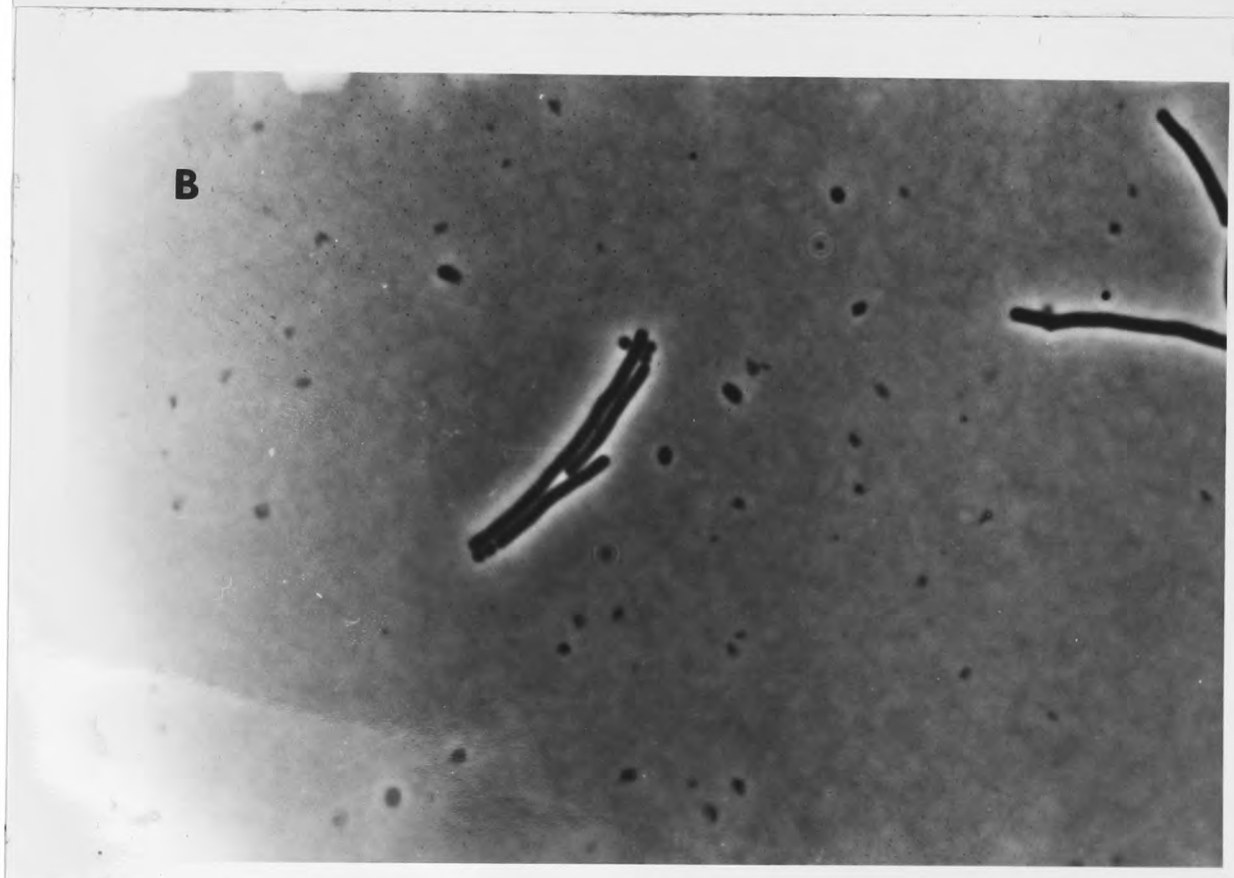
APPENDIX

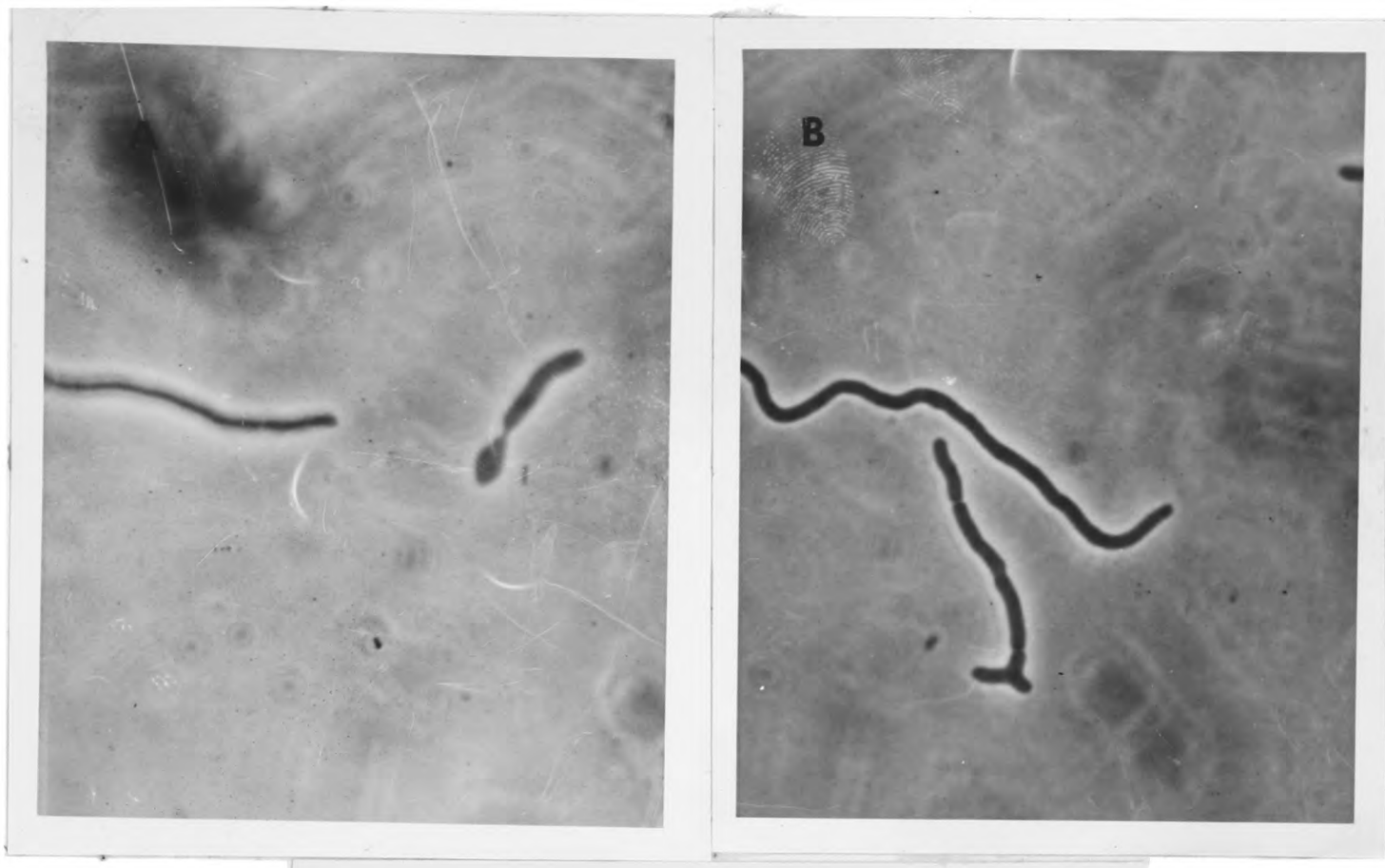
Photographs of the two mutants used in this study.

A. Filaments of lon⁻ cells induced by thymine starvation. The cells were starved of thymine for 60 min. When thymine was re-added to the medium, the cells were found to have lost the ability to divide. On the soft agar used to observe division in lon⁻ cells, the filaments typically formed coils of the kind seen here. Magnification about 2100X.

B. Minicells and 'mother cells' of a minicell producing strain. This picture shows the elongated state of the 'mother cell' and the very small size of the minicell. The cells in this field possess two minicells at one end. Magnification about 2500X.


A


B



Three examples of abnormal cells found after 180 min growth of lon cells in F-medium. A. An irregular shaped cell is seen dividing off from an elongated cell. B. A filament and a branched filament. The branched filament is separating into smaller cells. C. A branched filament showing branching at one end and in the middle of the cell. Also in the field is a more normal sized cell. All cells are at an approximate magnification of 1500X.

JOURNAL OF BACTERIOLOGY, June 1970, p. 810–814

Vol. 102, No. 3
Printed in U.S.A.

Deoxyribonucleic Acid Synthesis and Cell Division in a *lon*⁻ Mutant of *Escherichia coli*

P. M. LEIGHTON AND W. D. DONACHIE

MRC Molecular Genetics Unit, Department of Molecular Biology, University of Edinburgh, Edinburgh EH9 3JR, Scotland

JOURNAL OF BACTERIOLOGY, June 1970, p. 810-814
Vol. 102, No. 3

Deoxyribonucleic Acid Synthesis and Cell Division in a *lon*⁻ Mutant of *Escherichia coli*

P. M. LEIGHTON AND W. D. DONACHIE

MRC Molecular Genetics Unit, Department of Molecular Biology, University of Edinburgh, Edinburgh EH9 3JR, Scotland

Received for publication 17 February 1970

The *lon*⁻ mutants of *Escherichia coli* form long filamentous cells after temporary inhibition of deoxyribonucleic acid (DNA) synthesis by ultraviolet irradiation, treatment with nalidixic acid, or thymine starvation. The kinetics of DNA synthesis and cell division after a period of thymine starvation have been compared in *lon*⁺ and *lon*⁻ cells. After this treatment, both kinds of cells recover their normal DNA to mass ratio with the same kinetics. In contrast to previous reports, cell division is found to recommence in both *lon*⁺ and in *lon*⁻ cells after such a temporary period of inhibition of DNA synthesis. However, the delay separating the recommencement of DNA synthesis and of cell division is approximately three times as long in *lon*⁻ as in *lon*⁺ cells. Low concentrations of penicillin inhibit cell division in both *lon*⁺ and *lon*⁻ cells. In this case, cell division recommences with the same kinetics in both strains after the removal of penicillin. This suggests that different steps in the cell division process are blocked by inhibition of DNA synthesis and by penicillin treatment. The *lon*⁻ mutation appears to affect the former of these steps.

The *lon*⁻ mutants of *Escherichia coli* are a class of mutants which form filaments and fail to divide after ultraviolet (UV) irradiation (12). They are able to repair UV-induced lesions in their deoxyribonucleic acid (DNA; 12, 17), increase in mass, and synthesize DNA normally after UV (17). However, cell division is inhibited after irradiation, and the cells grow into long filaments (3). Filamentation is also induced by other agents which temporarily inhibit DNA synthesis (12, 18), and the effect of UV on cell division is, therefore, probably not a direct one but an indirect effect of temporary inhibition of DNA synthesis (18). In *lon*⁺ cells, inhibition of DNA synthesis also inhibits cell division (7), but in this case, cell division recommences in the treated cells as soon as the DNA to mass ratio in the cells is restored to its normal value (8, 9).

A possible explanation for the failure of *lon*⁻ populations to divide normally after inhibition of DNA synthesis, therefore, is that such populations do not recover a normal DNA to mass ratio. We therefore observed the course of DNA synthesis after a period of thymine starvation (or after UV irradiation) in *lon*⁺ and *lon*⁻ populations, to test whether the recovery of a normal DNA to mass ratio is delayed in the *lon*⁻ mutant. The kinetics of DNA synthesis after a period of inhibition appears to be the same in both mutant and wild-type, and both types of cells recover their DNA to mass ratio at the same time.

It has been reported that inhibition of cell division in *lon*⁻ cells can be prevented or reversed by a number of treatments, e.g., by "liquid holding" (13), by growth at 42 C (3, 5), or by a treatment with pantoyl lactone (2, 11, 16; *unpublished data*). A common result of these treatments is a decrease in the growth rate of the cells. It has also been reported (10) that cell division can be induced by a cell extract. This extract does not appear to inhibit growth (H. I. Adler, *personal communication*) and its action is so far not understood. Conversely, those treatments which increase the growth rate enhance the inhibition of cell division (3, 12, 18). In the present paper, we show that the inhibition of cell division in *lon*⁻ cells reverses spontaneously during post-treatment growth in the same medium. However, after restoration of the DNA to mass ratio, division is delayed longer in *lon*⁻ cells than in *lon*⁺ cells. The period of delay depends on the length of thymine starvation with a maximum delay of 120 to 140 min.

MATERIALS AND METHODS

Strains. *E. coli* AB1157 was obtained from H. I. Adler. The filamenting (*lon*⁻) derivative of this strain, *E. coli* AB1899NM (a nonmucoid mutant of the original *lon*⁻ isolate) was obtained from A. Hardigree, and

E. coli AB2497, a low-thymine (2 μg/ml)-requiring derivative of AB1157 was obtained from P. Howard-Flanders. The two isogenic strains used in these experiments were derived from AB1899NM. TG894, a low-thymine (2 μg/ml)-requiring derivative of the *lon*⁻ strain was obtained by treatment with trimethaprim (obtained through the courtesy of L. G. Petty, Burroughs Wellcome; reference 14). TG894LL, a *lon*⁺ derivative of TG894, was obtained by transduction with P1kc W3110 (from W. Brammar) and selection for *lac*⁺ colonies.

Media. Experiments were performed in minimal medium consisting of 300 ml of distilled water, 100 ml of M9 salts (4×; reference 1), $MgSO_4$ (10^{-3} M), glucose (0.2%), 8 mg of each of the required amino acids (arginine, histidine, threonine, leucine, and proline), and 8 mg of vitamin B_1. Where necessary, thymine (10 μg/ml) was added to the culture flasks. The L-agar used consisted of 1% tryptone, 0.5% yeast extract, 1% NaCl, 1.5% agar, and 1 liter of water. The L-soft agar for pour-plating the bacteria contained only 1.2% agar.

Radioactive counting. DNA synthesis was followed by the uptake of ^{14}C-thymine (The Radiochemical Centre, Amersham). ^{14}C-thymine (10 μg/ml, 0.1 μc/ml) was added to the medium, and 0.5-ml culture samples were collected into 0.5 ml of 20% trichloroacetic acid in ice. These were then filtered on Oxoid membrane filters (2 cm, 0.45 μm pores). The filters were washed with 5% trichloroacetic acid and dried under an infrared heat lamp. The dry filters were placed in vials containing 10 ml of 0.4% Scintillator *BBOT* (Ciba) in toluene. These were counted in a liquid scintillation counter (Beckman Instruments, Inc., Fullerton, Calif.).

Cell number. Increase in the cell number of a culture was followed by using an electronic counter (Coulter Electronics, Dunstable). Samples were taken into 10 ml of azide saline (0.9% NaCl, 0.08% NaN_3), and a 0.05-ml volume was counted. The penicillin (Solupen; Dista Products Ltd., Liverpool) was used in the experiments at a concentration of 15 μg/ml. The penicillinase (obtained from R. Ambler) was derived from *Staphylococcus aureus* P.C.1. Optical density was measured at 540 nm with a spectrophotometer (Hilger & Watts Ltd., London).

RESULTS

UV sensitivity. Testing sensitivity to UV irradiation was used to show these two isogenic strains were in fact *lon*⁻ and *lon*⁺. Figure 1 shows UV survival curves for these two strains (TG894 and TG894LL) and for the known *lon*⁻ and *lon*⁺ strains AB1899NM and AB1157. The derived strains showed the same UV-sensitivities as the original strains.

DNA synthesis after thymine starvation. It has been previously reported that there is a correlation between the restoration of the DNA to mass ratio of *lon*⁺ cells and the recommencement of cell division (9). A slower rate of recovery of the DNA content of *lon*⁻ cells could, therefore, lead to the increased delay in restoration of the ability to divide. We have, accordingly, compared the kinetics of DNA synthesis in TG894LL (*lon*⁺) and TG894 (*lon*⁻) cells after 80 min of thymine starvation. The course of DNA synthesis is identical in the two strains (Fig. 2). DNA synthesis continues at a decreasing rate for about 20 min after the removal of thymine from the medium and then stops, suggesting that, in this K-12 strain as in others (6), there is a pool of thymine in growing cells. DNA synthesis recommences as soon as thymine is added, and continues at a higher rate than in the unstarved controls, until the DNA content of the treated cells is restored almost to the level in the control cultures. The kinetics of DNA synthesis in these two K-12 strains are, therefore, similar to that described and discussed earlier for strain 15 (8).

The kinetics of DNA synthesis after UV irradiation have also been compared in TG894LL (*lon*⁺) and TG894 (*lon*⁻). In both *lon*⁺ and *lon*⁻ cells, DNA synthesis stopped after irradiation and recommenced at an accelerated rate after a delay. The normal DNA to mass ratio was restored at about the same time in both strains.

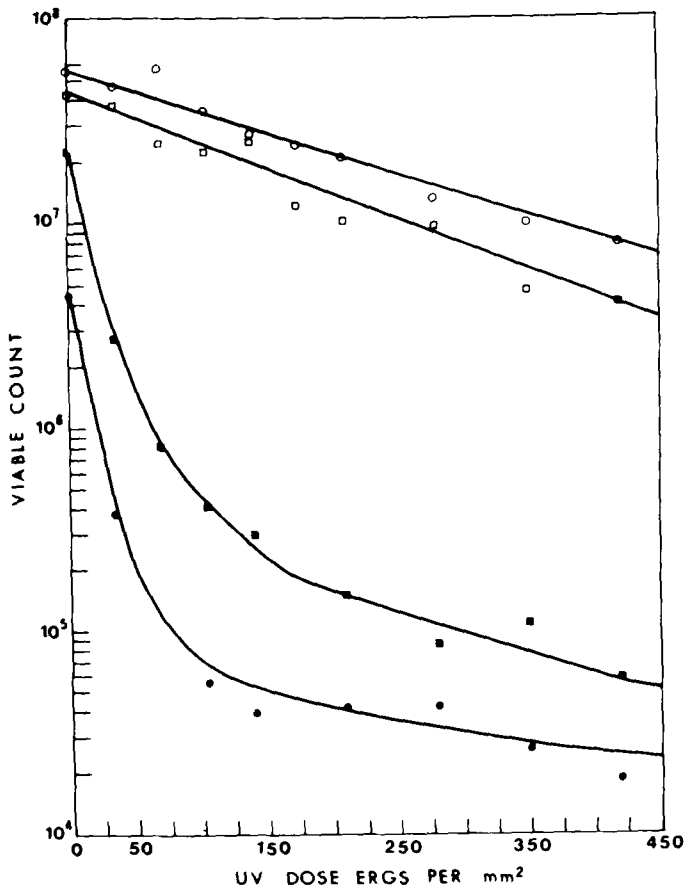


FIG. 1. *Viable count after UV irradiation. Cultures in the logarithmic phase of growth in minimal medium were irradiated. After irradiation, samples were pour-plated on L-agar containing added thymine (10 μg/ml) and incubated in the dark. AB1157 (lon⁺), □; AB1899 (lon⁻), ■; TG894LL (lon⁺), ○; and TG894 (lon⁻), ●.*

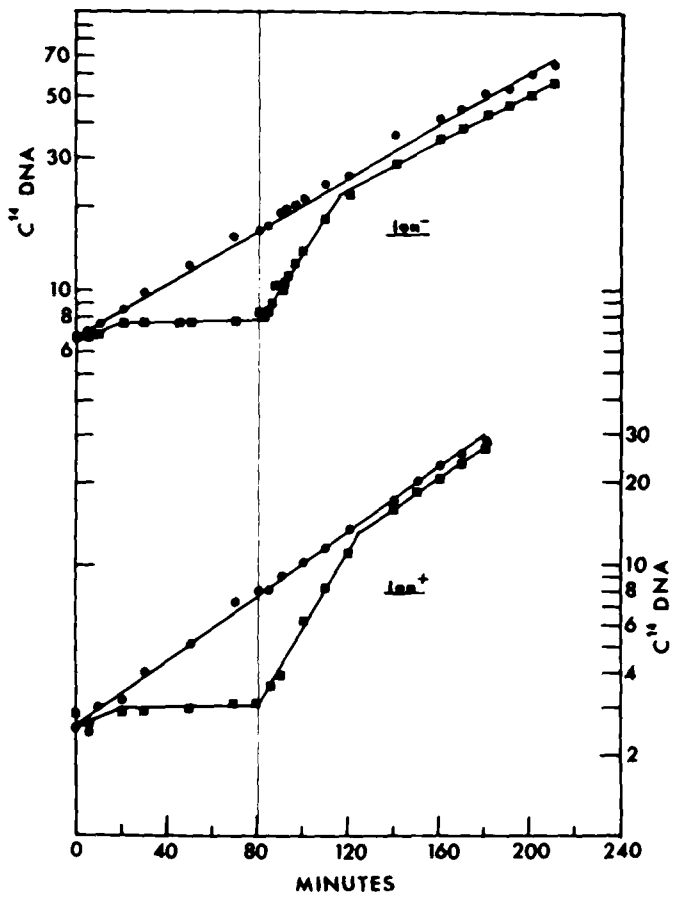


FIG. 2. *DNA synthesis in TG894LL (lon^+) and TG894 (lon^-) during and after thymine starvation. Cultures were grown for three mass doubling times in minimal medium containing ^{14}C-thymine. At 0 min, the culture was filtered, washed, and resuspended in fresh minimal medium containing ^{14}C-thymine (●), and in the absence of thymine (■). At 80 min, ^{14}C-thymine was added back to the starved cultures.*

Cell division after thymine starvation. Cultures of the thymine-requiring strains TG894LL (lon^+) and TG894 (lon^-) in the logarithmic phase of growth in minimal medium with thymine were grown in the absence of thymine by the method previously described (8). After 80 min, when the total cell mass (as measured by optical density) had increased about threefold, thymine was re-added to the thymine-starved cultures. Figure 3 shows the course of increase in total cell mass and in total cell number in such an experiment.

The cells continued to divide for some time after removal of thymine until the cell number had increased about 40%. After the readdition of thymine, the cell number remained constant for a period, and then began to increase again. The delay between the readdition of thymine (after 80 min starvation) and the recommencement of cell division was 40 to 50 min for lon^+ cells (8). However, the delay in the lon^- cells was consistently found to be 120 to 140 min (Fig. 3). Apart from this difference in the length of the delay period, the kinetics of division on the lon^- cell appears to be the same as previously reported for lon^+ strains (8). Similar results were obtained when the experiment was performed in an enriched medium.

As previously reported (8), the length of the delay between readdition of thymine and recommencement of division is a function of the length of the preceding period of growth without thymine. The results with the lon^+ strain AB2497 used in the present work (Fig. 4) are closely similar to those reported earlier. However, the results for the lon^- strain, TG894, show a much more rapid increase in the length of delay of cell division after thymine starvation. Also, the maximal length of the delay is 120 to 140 min (as opposed to 45 min for the lon^+ strain). This means that the lon^- cells show about three times the maximal delay before cell division shown by

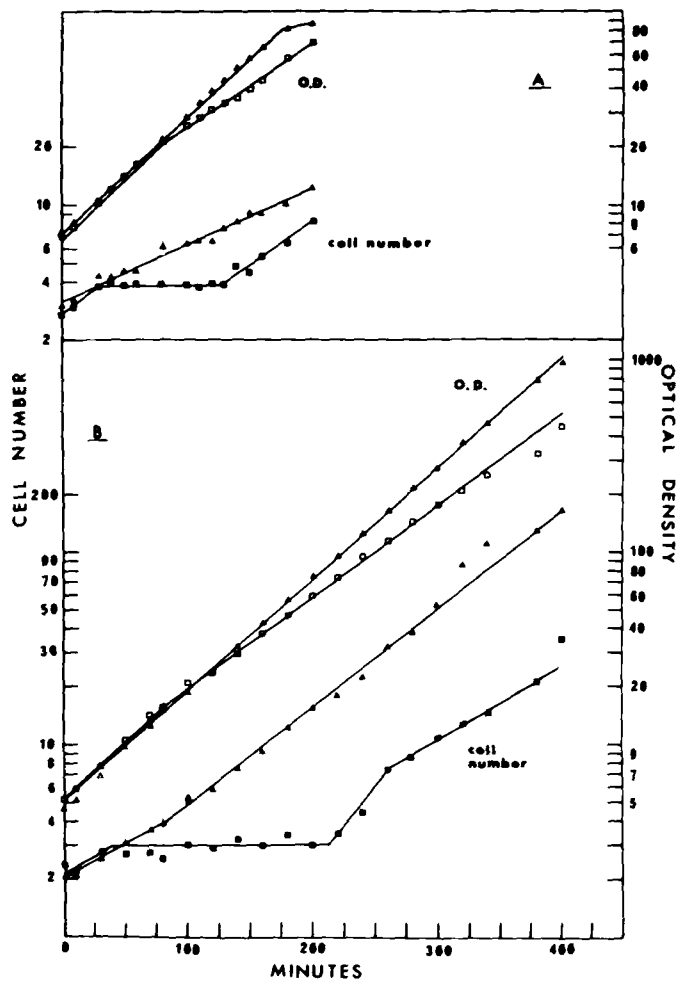


FIG. 3. *Cell number in TG894LL (lon^+) and TG894 (lon^-) during and after thymine starvation. At 0 min, log-phase cultures growing in minimal medium were filtered, washed, and resuspended in the presence (▲, △) and absence (■, □) of thymine (solid symbols, cell number; open symbols, optical density). At 80 min, thymine was added back to the starved cultures. B, To maintain the culture in logarithmic growth, the thymine-starved culture was diluted into fresh warm medium at 80 and 250 min, and the control unstarved culture was similarly diluted at 80, 250, and 300 min. The graph has been corrected for these dilutions. A, lon^+; B, lon^-.*

the *lon*⁺ cells, although both exhibit this maximal delay after starvation for the same length of time, 60 min. The length of the delay before cell division is dependent (up to a maximum) on the length of the period of inhibition of DNA synthesis for both *lon*⁺ and *lon*⁻ strains.

Cell division after penicillin treatment. Thymine starvation results in a cessation of DNA synthesis, and subsequent to this, an inhibition of cell division. The *lon*-induced filamentation could be due to inhibition of DNA synthesis or to the subsequent inhibition of the cell division process itself. If the latter is the case, inhibition of cell division without affecting DNA synthesis should also result in filamentation. To test this hypothesis, we inhibited cell division with low concentrations of penicillin, which inhibit cell division without altering the rate of growth or DNA synthesis (15). Therefore, in the presence of such a concentration of penicillin, cells grow into long filaments. The removal of penicillin from the medium (e.g., by the addition of penicillinase) leads to a resumption of cell division in wild-type cells (15).

The kinetics of cell division after penicillin filamentation was compared in *lon*⁺ and *lon*⁻ cells. Figure 5 shows that, in this case, the kinetics of cell division in the wild type and in the mutant are identical. Both cultures recommenced cell division at the same rate, about 45 min after the addition of penicillinase.

Figure 5 shows the combined effect of penicillin and thymine starvation. At zero minutes, thymine was removed and penicillin was added. At 80 min, thymine and penicillinase were added together. The effect of the combined treatment is the same as with thymine starvation alone.

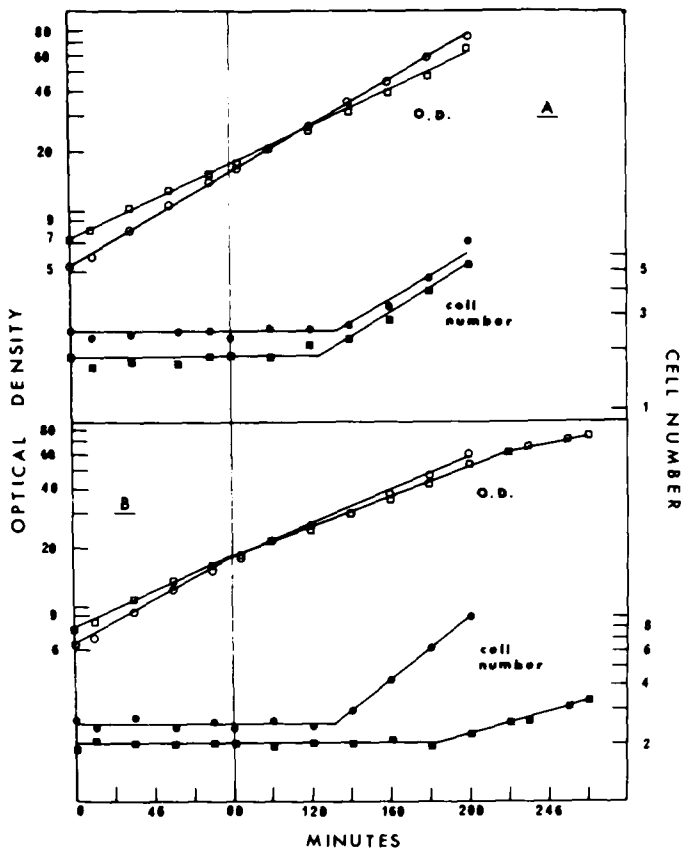


FIG. 5. *Effect of penicillin (A) and penicillin with thymine starvation (B) on cell division in TG894 (lon⁻, ■, □) and TG894LL (lon⁺; ●, ○); solid symbols = cell number, open symbols = optical density. At 0 min, penicillin (15 μg/ml) was added to the cultures (A), or the cultures were filtered, washed, and resuspended in minimal medium containing penicillin (15 μg/ml) in the absence of thymine (B). At 80 min, penicillinase (0.3 μg/ml; A), or penicillinase (0.3 μg/ml) with thymine (10 μg/ml; B) was added to the cultures.*

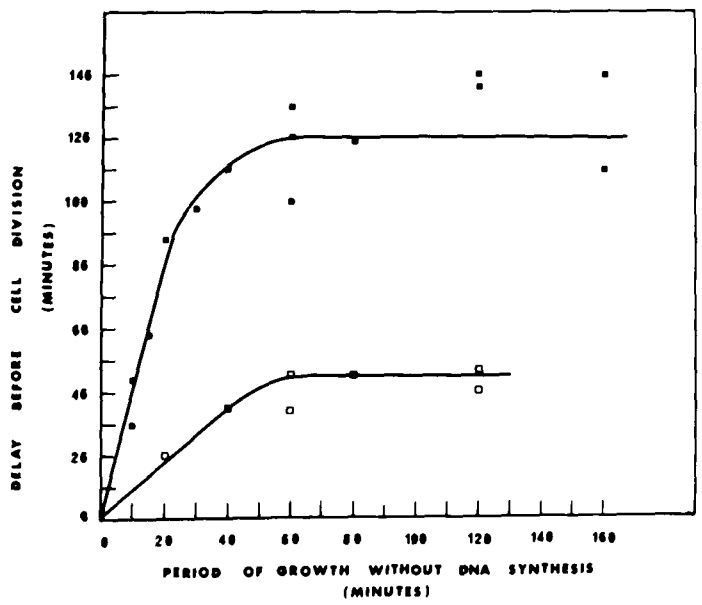


FIG. 4. *Relationship between pregrowth in the absence of DNA synthesis (thymine starvation) and the delay before cell division after DNA synthesis recurs (readdition of thymine). To allow for the thymine pool, the length of time of no DNA synthesis is taken as the time of thymine starvation less 20 min. TG894 (lon⁻), ■; AB2497 (lon⁺), □.*

DISCUSSION

The experiments described here were performed to test the possibility that the inhibition of cell division in *lon*⁻ cells, which follows thymine starvation, is due to an inability of the cells to restore their normal DNA to mass ratio after a temporary inhibition of DNA synthesis. Such a failure might have arisen from a defect in the normal regulation of DNA synthesis, such as, for example, the synthesis of an unstable initiator protein in the mutant. However, the regulation of DNA synthesis in this mutant appears to be normal, at least with respect to the kinetics of DNA synthesis after thymine starvation or UV irradiation. The mutant defect, however, clearly involves those steps which link DNA replication to cell division. Thus, treatments which temporarily inhibit cell division without interfering with

DNA synthesis have exactly the same effects in *lon*$^+$ and *lon*$^-$ cells, whereas any treatment which temporarily inhibits DNA replication inhibits cell division for a much longer period in *lon*$^-$ than in *lon*$^+$ cells.

Previous reports have suggested that filamentation in *lon*$^-$ cells, once induced, is irreversible except by special treatments which generally have the effect of slowing or preventing cell growth (3, 4). The experiments described here were carried out on continuously growing log-phase cells in liquid minimal medium. They have shown that, in fact, cell division resumed in *lon*$^-$ as in *lon*$^+$ cells after temporary inhibition of DNA synthesis. The difference between mutant and wild-type appears to lie in the length of the delay between restoration of the normal DNA to mass ratio and recommencement of cell division. Thus, in the experiments shown in Fig. 3, cell length (taken to be proportional to optical density) increased about 4-fold in the *lon*$^+$ strain, and by 15-fold in the *lon*$^-$ strain before cell division recommenced.

The data plotted in Figure 3 suggest that, as in other strains (8), cell division is restricted to an average of one per cell during the first period of rapid division. Such a restriction must lead to a perpetuation of the filaments in the culture. More detailed observations of the exact pattern of division in these cells are also in progress. Preliminary experiments also show that the filaments themselves do divide, and the increase in cell numbers is not due only to the division of cells of normal length in the culture.

ACKNOWLEDGMENTS

P. M. L. was the recipient of a Wellcome Research Training Scholarship.

We thank Millicent Masters for many helpful discussions during this work and in the preparation of this paper.

LITERATURE CITED

1. Adams, M. H. 1959. Bacteriophages. Interscience, New York.
2. Adler, H. I., and A. A. Hardigree. 1964. Analysis of a gene controlling cell division and sensitivity to radiation in *Escherichia coli*. J. Bacteriol. 87:720–726.
3. Adler, H. I., and A. A. Hardigree. 1965. Postirradiation growth, division and recovery in bacteria. Radiat. Res. 25:92–102.
4. Adler, H. I., and A. A. Hardigree. 1965. Growth and division of filamentous forms of *Escherichia coli*. J. Bacteriol. 90:223–226.
5. Anderson, E. H. 1951. Heat reactivation of ultraviolet-irradiated bacteria. J. Bacteriol. 61:389–394.
6. Caro, L. G., and G. M. Berg, 1968. Chromosome replication in some strains of *Escherichia coli* K12. Cold Spring Harbor Symp. Quant. Biol. 33:559–573.
7. Cohen, S. S., and H. D. Barner. 1954. Studies on unbalanced growth in *Escherichia coli*. Proc. Nat. Acad. Sci. U.S.A. 40:885–893.
8. Donachie, W. D. 1969. Control of cell division in *Escherichia coli:* experiments with thymine starvation. J. Bacteriol. 100:260–268.
9. Donachie, W. D., D. G. Hobbs, and M. Masters. 1968. Chromosome replication and cell division in *Escherichia coli* 15T$^-$ after growth in the absence of DNA synthesis. Nature (London) 219:1079–1080.
10. Fisher, W. D., H. I. Adler, F. W. Shull, Jr., and A. Cohen. 1969. Properties of a cell fraction that repairs damage to the cell division mechanism of *Escherichia coli*. J. Bacteriol. 97:500–505.
11. Grula, E. A., and M. M. Grula. 1962. Cell division in a species of *Erwinia*. III. Reversal of inhibition of cell division caused by D-amino acids, penicillin and ultraviolet light. J. Bacteriol. 83:981–988.
12. Howard-Flanders, P., E. Simson, and L. Theriot. 1964. A locus that controls filament formation and sensitivity to radiation in *Escherichia coli* K12. Genetics 49:237–246.
13. Roberts, R. B., and E. Aldous. 1949. Recovery from ultraviolet irradiation in *Escherichia coli*. J. Bacteriol. 57:363–375.
14. Stacey, K. A., and E. Simson. 1965. Improved method for the isolation of thymine-requiring mutants of *Escherichia coli*. J. Bacteriol. 90:554–555.
15. Starka, J., and J. Moravova. 1967. Cellular division of penicillin-induced filaments of *Escherichia coli*. Folia Microbiol. 12:240–247.
16. van de Putte, P., C. Westenbroek, and A. Rörsch. 1963. The relationship between gene-controlled radiation resistance and filament formation in *Escherichia coli*. Biochim. Biophys. Acta 76:247–256.
17. Walker, J. R., and A. B. Pardee. 1967. Conditional mutations involving septum formation in *Escherichia coli*. J. Bacteriol. 93:107–114.
18. Walker, J. R., and A. B. Pardee. 1968. Evidence for a relationship between deoxyribonucleic acid metabolism and septum formation in *Escherichia coli*. J. Bacteriol. 95:123–131.